

Investigating the functions of small non-coding RNAs in stolon-to-tuber transition stages of potato development

विद्या वाचस्पति की
उपाधि की अपेक्षाओं की आंशिक पूर्ति में प्रस्तुत शोध प्रबंध

A thesis
submitted in partial fulfillment of the requirements

of the degree of
Doctor of Philosophy

द्वारा /By

मालणकर निलम नामदेव नमिता / Malankar Nilam Namdeo Namita

पंजीकरण सं. / Registration No.:
20142002

शोध प्रबंध पर्यवेक्षक / Thesis Supervisor:
प्रो. अंजन के. बैनर्जी / Prof. Anjan K. Banerjee



भारतीय विज्ञान शिक्षा एवं अनुसंधान संस्थान पुणे
INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

2023

DECLARATION

I declare that this written submission represents my ideas in my own words and where other's ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.



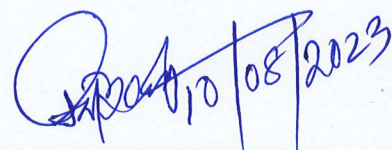
Nilam Namdeo Malankar

Reg. No. 20142002

Date: 10th August, 2023

CERTIFICATE

Certified that the work incorporated in the thesis entitled “**Investigating the functions of small non-coding RNAs in stolon-to-tuber transition stages of potato development**” submitted by **Ms. Nilam Namdeo Malankar** (Reg. No. 20142002) was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other university or institution.



Prof. Anjan K. Banerjee

Supervisor

Date: 10th August, 2023

प्रो. अंजन के. बॅनर्जी/Prof. Anjan K. Banerjee
प्राध्यापक, जीवशास्त्र कार्यक्रम
Professor, Biology Program
भारतीय विज्ञान शिक्षा एवं अनुसंधान संस्थान
Indian Institute of Science Education & Research
पुणे, भारत /Pune- 411 008, India

Acknowledgements

I want to thank my supervisor, Prof. Anjan Kumar Banerjee, for his constant support throughout my PhD. He always encouraged me to pursue scientific approaches and tackle problems. His enthusiasm towards science and tireless work style has inspired me to do the same.

I want to thank my RAC members Prof. P. V. Shivaprasad from NCBS Bangalore and Dr. Krishanpal Karmodiya from IISER Pune for their valuable suggestions throughout my PhD journey. Their timely and insightful suggestions have helped me in shaping the research work presented in this thesis.

I want to thank IISER Pune for providing research infrastructure to work, and financial support given during the early days of joining the course. I thank CSIR (JRF and SRF) for providing fellowship during the later years of my course. I want to acknowledge the funding from Department of Biotechnology (DBT) and Science and Engineering Research Board (SERB), Government of India towards my projects.

*My sincere gratitude towards fellow scientists for sharing essentials, transgenic lines and biological reagents that were a tremendous help in pursuing my research objectives. I would like to thank Prof. (Sir) David Baulcombe, University of Cambridge, UK, for providing seeds of RDR6i mutant lines of *Nicotiana Benthamiana*. My sincere thanks to Prof. Peter Hedden for providing Gibberellin standards (GA₁ and GA₄) for LC-MS/MS experiments. I want to thank Dr. Jyothilakshmi Vadassery, NIPGR, and NIPGR Metabolomics Facility, India for conducting Gibberellin quantifications from potato plants as a part of this study.*

I would like to thank all Past and current Molecular Plant Biology lab members (“The red background group”) for keeping lively lab environment, that helped me to perform efficiently in the lab. I, especially want to thank Dr. Kirtikumar Kondhare, Faculty, CSIR-NCL Pune (former post-doc of our lab) for his significant contribution in shaping my research aptitude and mentoring me throughout my PhD. Dr. Ravi Devani helped me to learn RNAseq analysis. I, greatly acknowledge the help from Kishan, Mohit, Tanvi, Zahra, Arghyadeep and Udaat to my research. A special thanks to Harpreet and Nikita for all those fun-filled and stress-free scientific and non-scientific discussions that resulted in my improved understanding towards science and life. I want to thank my lab seniors Bhavani, Boomi, Ravi, Amit, Harpreet, and Amey for their help in conceptualizing plant science and for critical comments on my work. I want to thank Vyankatesh, Sukanya, Anindita, Apurva, Sheeba, Arati, Pallavi, Shirsa, and

Sneha for being my Chai Partners and for healthy discussions during tea breaks. I want to thank SG, Krish, and SP lab members for keeping a cheerful atmosphere around the lab. I thank Mr. Nitish and Mr. Niles, our greenhouse maintenance staffs for taking care of the growth and development of the plants. I acknowledge a great help from Biology admin staff Kalpesh, Piyush, Sandeep, Mahesh and Hariprasad, as well as, IISER admin staff Tushar, Sayalee, and Sachin with all the necessities during PhD.

I want to thank all my friends from IISER Pune, for making me feel like a part of big family. First I want to thank my seniors Mukul, Divya, Shivani, Swathi, Dhriti and Amar for guiding me during lab hunting in my initial years and their stress-bursting conversations. I would like to thank my Integrated PhD (2014) batch-mates, Shikha, Kriti, Mayur, Deepak K, Yashaswi, Saurabh, Shalini, Punita, Dipti, Ankita, Rajesh, Yashwant and Deepak S for all the fun-filled, wonderful memories we created together that I will cherish forever.

A special thanks to Harpreet, my senior for constantly pouring friendship, love, and care. He stood up with me during our stay at IISER during the COVID phase. I am blessed to gain Shikha and Kriti as my life-long friends on this journey. A heartfelt thanks to these girls who filled my PhD life with laughter, fun, and happiness. I will cherish this time we spent together with dinner outings, shopping, trips, breakfast talks, and lounging around. A special thanks to Shikha, for being there with me in my PhD final year, and supporting me through all rough times.

Lots of thanks to my bestie, Payal Pawar for staying in touch with me throughout the PhD journey. I want to thank her for supporting my dreams, for protecting me, putting up with my anger and terrible jokes, and caring for me. I would like to thank my parents Mr. Namdeo and Mrs. Namita for encouraging me to pursue my dream, and constant support with their love. I thank my siblings Namrata and Niles for sharing a number of smiles, laughs, thoughts and tears of my life. I want to give all my love to my niece Mauli.

Love you all.

Nilam Namdeo Malankar

20142002

Table of Contents

	Contents	Page no.
	<i>List of Figures</i>	1
	<i>List of Tables</i>	5
	<i>Abbreviations</i>	6
	<i>Synopsis</i>	9
Chapter 1: Introduction		
1.1.	Potato	17
1.2.	Tuberization	17
1.2.1.	Stolon-to-tuber transitions	18
1.2.2.	Environmental Factors that influence tuber formation	19
1.2.2.1.	Role of photoperiod	19
1.2.2.2.	Role of temperature	20
1.2.2.3.	Effect of Nitrogen levels	20
1.3	Hormonal regulation of tuber formation	21
1.3.1	Gibberellin (GA)	21
1.3.2	Auxin	22
1.3.3.	Cytokinin	22
1.3.4.	Strigolactones	23
1.3.5	Jasmonic acid	23
1.3.6.	Absciscic acid	23
1.3.7.	Gibberellin (GA) and auxin cross talk during tuber development.	24
1.4	Molecular mechanism of photoperiod dependent tuberization	25
1.4.1	Mobile signals in tuber development	26
1.4.2	StBEL5-StKNOX heterodimer complex mediate tuber development	27
1.4.3.	Tuber activation complex (TAC)	28
1.5	Small RNAs in plant development	29
1.5.1	Biogenesis of miRNAs and phasiRNAs	29
1.5.2.	Mode of action	30

1.6	Functions of miRNAs in plant growth and development	31
1.7	MiRNAs regulating tuber development	33
1.7.1.	miRNA172: a positive regulator of tuberization	33
1.7.2.	miRNA156: an inducer of aerial tubers in potato	34
1.7.3.	miRNA390 in tuber development	34
1.7.4	SES-SP6A regulation in tuber formation	35
1.8	PhasiRNA pathways in plant development and defense responses	35
1.8.1	Conserved miR390-TAS3-ARF3 module regulate plant development	35
1.8.2	miR828-TAS4-MYB module in anthocyanin biosynthesis	36
1.8.3	miR9678-WSGAR module in seed germination	37
1.9	PhasiRNAs in <i>solanaceae</i> family	38
1.9.1	miR482c/miR2118 family regulates NB-LRR genes in <i>solanaceae</i>	38
1.9.2	PhasiRNA-gibberellin module in potato during PVY infection	39
1.10	Identification of small RNAs from potato: previous studies.	39
1.11	<i>Solanum tuberosum</i> ssp. <i>andigena</i> as a model system	40
1.12	Hypothesis and Objectives	40
Chapter 2: Identification of small RNAs and target genes from stolon-to-tuber transition stages of potato under photoperiodic conditions		
2.1.	Introduction	44
2.2.	Material and methods	46
2.2.1.	Plant growth conditions and stolon sample harvest	46
2.2.2.	Processing of raw reads and miRNA predictions	46
2.2.3.	Differential expression analysis for miRNAs	47
2.2.4.	Identification of putative miRNA targets and Gene ontology	47
2.2.5.	Cleavage site mapping	47
2.2.6.	cDNA synthesis and quantitative PCRs	48
2.2.7.	PhasiRNA predictions and their putative targets	48
2.2.8	Validations of <i>TAS –like loci</i>	49
2.2.9	Accession numbers	49
2.2.10.	Primer details	50

2.3.	Results	52
2.3.1.	Small RNA expression profile from stolon samples	52
2.3.2.	Identification of conserved and potato specific miRNAs	54
2.3.3.	Differential expression (DE) analysis for miRNAs	55
2.3.4.	Target predictions and GO analysis	61
2.3.5.	Time course expression analysis for miRNA-target pairs	62
2.3.6.	Cleavage site mapping for targets of conserved miRNA through 5' RLM-RACE	63
2.3.7.	Identification of phased siRNAs	64
2.3.8	Validation of TAS like loci and putative phasiRNAs	67
2.3.9	Conserved TAS loci in potato	68
2.3.10	Putative phasiRNAs that could regulate key tuberization genes	69
2.4.	Discussion	70
2.4.1.	Photoperiod influences miRNA expressions during early stages of stolon-to-tuber transitions	70
2.4.2.	DE miRNA and their targets exhibits potential role during tuber development	71
2.4.3.	PhasiRNAs: A new addition to gene regulatory pathways in tuber development	72
Chapter 3: PhasiRNA siRD29(-) regulates a gibberellin biosynthesis gene <i>StGA3ox3</i> during stolon-to-tuber transitions in potato		
3.1	Introduction	77
3.2	Materials and methods	79
3.2.1	Plant materials and growth conditions	79
3.2.2.	Gene expression analysis	79
3.2.3.	Cleavage site mapping by 5' RLM-RACE assay	80
3.2.4.	Phylogenetic analysis	80
3.2.5.	Gene constructs designs and generation of potato transgenic lines	80
3.2.6.	<i>Agrobacterium</i> mediated co-infiltration assays	81
3.2.7.	Dot blot analysis	82
3.2.8.	Analysis of <i>StGA3ox3</i> promoter activity	82

3.2.9.	Histology and microscopy	83
3.2.10.	Hormone treatment	83
3.2.11.	Estimation of chlorophyll and anthocyanin content	83
3.2.12.	<i>In vitro</i> tuberization	84
3.2.13	Small RNA sequencing	84
3.2.14	Processing of sRNA reads for the identification of phasiRNAs	84
3.2.15.	Quantification of GAs from <i>Agrobacterium</i> -infiltrated potato leaves	85
3.2.16.	Gibberellin (GAs) quantification from transgenic lines	85
3.2.17.	Grafting	86
3.2.18.	Accession numbers	86
3.2.19.	Primer Sequences	87
3.3.	Results	89
3.3.1.	MicroRNA482c cleaves <i>StGA4</i> , a PHAS locus encoding NB-ARC domain-containing disease resistance protein in potato	89
3.3.2.	SiRD29(-) generation is RDR6-dependent	89
3.3.3.	A novel phasiRNA siRD29(-) targets <i>StGA3ox3</i>	91
3.3.4	Stu-miR482c triggers secondary siRNAs from <i>StGA4</i> locus <i>in planta</i>	93
3.3.5	<i>StGA3ox3</i> is highly expressed in the swollen stolon under SD	94
3.3.6	Altered expression of <i>StGA3ox3</i> affects leaf size and chlorophyll content with no effect on tuber weight	97
3.3.7	<i>StGA4</i> overexpression lines have high levels of siRD29(-) and mimic the tuber phenotype of <i>StGA3ox3</i> -AS lines	101
3.3.8	<i>StGA3ox3</i> controls tuber-stalk development under <i>in vitro</i> conditions	104
3.3.9	Altered expression of <i>StGA3ox3</i> affect GA levels in transgenic lines	107
3.3.10	Promoter of <i>StGA3ox3</i> gene has phyto-hormone responsive motifs.	108
3.3.11	StBEL5 (a BEL-like TF) induces <i>StGA3ox3</i> expression in leaves of <i>promStGA3ox3::GUS</i> lines	110
3.4.	Discussion	111
3.4.1.	Novel Stu-miR482c- <i>StGA4</i> -siRD29(-)- <i>StGA3ox3</i> GRM is functional in potato	111
3.4.2.	StBEL5 regulates <i>StGA3ox3</i> expression in potato	114

3.4.3.	Dual regulation of <i>StGA3ox3</i> during stolon-to-tuber transitions	115
3.5	Availability of raw sequencing data	118
Chapter 4: Understanding the function of potato specific miRNA72 to regulate tuber formation in potato		
4.1.	Introduction	123
4.2.	Material and methods	125
4.2.1.	Plant growth and conditions	125
4.2.2.	Validation of miRNA72 in potato	125
4.2.3.	Bioinformatic prediction and cleavage site mapping analysis for miR72 targets	125
4.2.4.	Expression analysis of miR72	126
4.2.5.	Gene construct designs and transgenic lines generation	126
4.2.6.	Northern blot for the detection of miR72	127
4.2.7.	Primer Details	128
4.2.8.	Accession number	128
4.3.	Results	129
4.3.1.	Validation of potato specific miRNA72 in potato	129
4.3.2.	Abundant expression of miR72 in different tissue types of potato	130
4.3.3.	Overexpression (OE) of miR72 affects tuber and root development, and leads to sessile tuber formation in potato	131
4.3.4.	miR72 targets interesting candidate genes	134
4.4.	Discussion	136
4.4.1.	Potato specific miRNA, miR72 expression reduces in tuberizing stolons.	136
4.4.2.	Altered expression of miR72 affects tuber and root development in transgenic lines.	137
4.4.3.	Altered expression of miR72 induces belowground sessile tuber formation	138
	Summary and future directions	141
	References	148
	Annexure I	159

List of Figures

Chapter 1: Introduction

1.1	Pictorial representation of potato plant showing stolon-to-tuber transitions	18
1.2	Timeline for tuber developmental stages of potato.	19
1.3	GA and IAA regulation at stolon tips during stolon-to-tuber transitions.	24
1.4	Photoperiod dependent tuberization pathway in andigena potato plant.	27
1.5	miRNA and phasiRNA biogenesis.	30
1.6	miRNAs regulate SAM and leaf development	32
1.7	miRNA module in flower development.	33
1.8	TasiRNA mediated leaf polarity establishment	36
1.9	PhasiRNAs in plant development and stress responses	38
1.10	<i>Solanum tuberosum</i> ssp. andigena: a model potato plant	40

Chapter 2: Identification of small RNAs and target genes from stolon-to-tuber transition stages of potato under photoperiodic conditions

2.1	Schematic representation of experimental design.	53
2.2	Size distribution profile of small RNA sequences identified in sRNA libraries.	53
2.3	Comparison for sRNA analysis using miRPro between small RNA sequencing from the lab and previously available dataset.	55
2.4	MA scatter plot analysis for conserved and novel miRNAs.	56
2.5	MA scatter plot analysis for DE miRNAs from Zhang et al., 2013.	56
2.6	Heat map clustering for (A) Conserved miRNAs and (B) novel miRNAs from stolon libraries.	57
2.7	Validation of sRNA sequencing data by quantitative PCR.	59
2.8	Expression analysis of differentially expressed conserved miRNAs in stolons under LD vs SD photoperiod conditions	60
2.9	Expression analysis of Differentially expressed Novel miRNAs in stolons under LD vs SD photoperiod conditions.	60

2.10	Gene Ontology (GO) categorization for predicted targets of identified novel and conserved miRNAs.	62
2.11	Relationship between miRNAs and their predicted targets by qRT-PCR analysis at 4 d time-point under LD vs SD photoperiod conditions.	63
2.12	5' RLM-RACE for cleavage site mapping	64
2.13	Number of <i>TAS-like</i> loci identified using different parameters (p values).	64
2.14	Graphical representation of StTm2 and StPHO2 <i>TAS-like</i> loci and the most abundant siRNAs generated from these two loci	67
2.15	Alignment of TAS3 and TAS5 genes from <i>Solanaceae</i> family	69

Chapter 3: PhasiRNA siRD29(-) regulates a gibberellin biosynthesis gene *StGA3ox3* during stolon-to-tuber transitions in potato

3.1	A phasiRNA siRD29(-) generates from <i>StRGA4</i> transcript.	90
3.2	Phylogenetic relationship of GA3ox proteins.	91
3.3	siRD29(-) targets <i>StGA3ox3</i> mRNA post-transcriptionally	92
3.4	Expression of siRD29(-) in potato tissue types	92
3.5	A phasiRNA siRD29(-) is generated from the antisense strand of <i>StRGA4</i> transcript at the 29 th phase post Stu-miR482c-mediated cleavage.	94
3.6	Expression analysis of <i>StGA3ox3</i> in in different tissues of potato cultivar andigena	95
3.7	Expression analysis of GA metabolism genes during stolon-to-tuber transitions under SD.	96
3.8	<i>StGA3ox3</i> promoter exhibits tissue-specific GUS activity.	97
3.9	Overexpression (OE) and knockdown (AS) lines of <i>StGA3ox3</i> .	98
3.10	Overexpression of <i>StGA3ox3</i> affects plant height and leaf size	99
3.11	<i>StGA3ox3</i> overexpression affects chlorophyll and anthocyanin content of leaves	99
3.12	Knockdown of <i>StGA3ox3</i> alters tuber numbers in potato	100
3.13	Tuber phenotype in grafts generated from <i>StGA3ox3</i> OE, AS lines and WT	101
3.14	Overexpression lines of <i>StGA3ox3</i> and <i>StRGA4</i> have enhanced levels of siRD29(-)	102

3.15	<i>StRGA4-OE</i> lines exhibits phenotype similar to <i>StGA3ox3-AS</i> lines with reduced tuber number	103
3.16	<i>StRGA4-OE</i> lines have enhanced levels of siRD29(-) in stolons.	103
3.17	Quantification of GA ₁ levels from leaves of potato plant infiltrated with <i>Agrobacterium</i> containing 35S:: <i>StGA3ox3</i> gene construct.	104
3.18	Rate of tuber formation in transgenic lines during <i>in vitro</i> tuberization assay	105
3.19	Altered <i>StGA3ox3</i> expression influences development of tuber-stalk during <i>in vitro</i> tuberization assays.	105
3.20	Effect of GA inhibitor treatment on <i>StGA3ox3-OE</i> lines during <i>in vitro</i> tuberization assay	106
3.21	Effect of <i>StGA3ox3</i> expression on stolon length in soil-grown plants.	107
3.22	<i>StGA3ox3</i> promoter is enriched with hormone-inducible motifs.	109
3.23	Effects of phytohormone treatments on <i>StGA3ox3</i> expression	109
3.24	<i>StGA3ox3</i> promoter is enriched with StBEL5 binding site motifs	110
3.25	BEL1-like TF StBEL5 regulates the expression of <i>StGA3ox3</i> .	111
3.26	Expression of <i>StGA3ox2</i> transcript in <i>StGA3ox3</i> OE, AS and <i>StRGA4</i> OE lines.	112
3.27	Proposed model for the dual regulation of <i>StGA3ox3</i> during stolon-to-tuber transitions of potato.	116
3.28	Conservation of stu-miR482c- <i>StRGA4</i> -siRD29(-)- <i>StGA3ox3</i> gene regulatory module in tomato.	117

Chapter 4: Understanding the function of potato-specific miRNA72 to regulate tuber formation in potato

4.1	Detection of potato-specific miRNAs and their expression analysis in stolons under photoperiodic conditions.	129
4.2	Detection of miR72 and its precursor from short day (SD) induced leaves and their confirmation by Sanger's sequencing.	130
4.3	Intronic miR72 exhibits tissue-specific expression pattern and reduces during stolon developmental stages.	131
4.4	MiRNA72 overexpression affects leaf architecture in transgenic lines.	132

4.5	MiRNA72 overexpression showed moderate phenotype for leaf architecture in transgenic lines.	133
4.6	Overexpression of miRNA72 affects tuber and root development in transgenic lines	134
4.7	miR72-OE lines exhibited belowground sessile tuber formation.	135
4.8	Knockdown lines generation using STTM approach and cleavage site mapping assay for miR72 targets.	135

List of Tables

Chapter 2: Identification of small RNAs and target genes from stolon-to-tuber transition stages of potato under photoperiodic conditions

2.1	Summary table for small RNA sequencing studies from potato.	45
2.2	Accession numbers	49
2.3	List of primers	50
2.4	Summary of reads obtained from stolon small RNA libraries.	54
2.5	List of differentially expressed conserved as well as novel miRNAs identified from LD4 vs SD4 and LD10 vs SD10 stolon transition comparisons.	58
2.6	List of <i>TAS-like</i> loci predicted to be cleaved by conserved or novel miRNAs.	65
2.7	Putative <i>TAS-like</i> loci and miRNAs targeting these loci for siRNA generation that could regulate key tuberization genes	74

Chapter 3: PhasiRNA siRD29(-) regulates a gibberellin biosynthesis gene *StGA3ox3* during stolon-to-tuber transitions in potato

3.1	Accession numbers	86
3.2	List of primers	87
3.3	Levels of GAs (ng/g of freeze-dried tissue weight) in the shoot-apices of the transgenic lines	108
3.4	Small RNA sequencing of stolon-to-tuber transitions stages from andigena and identification of <i>TAS-like</i> loci - <i>StGA4</i> .	118
3.5	List of siRNAs generated from <i>StGA4</i> locus, post miR482c cleavage	119
3.6	List of motifs present with 2.2 kb upstream sequence from TSS of <i>StGA3ox3</i> gene	121

Chapter 4: Understanding the function of potato specific miRNA72 to regulate tuber formation in potato

4.1	Primer List	128
4.2	Accession list	128
4.3	<i>In silico</i> target prediction for miR72 in potato	139

Abbreviations

4-Methylumbelliferyl- β -D-glucuronide hydrate	MUG
Anti-sense	AS
APETELLA 2	AP2
ARGONAUTE	AGO
Auxin Response Factors	ARF
Base Pairs	bp
Branched 1b	BRC1b
Brassinosteroid Insensitive 1	BRI1
Brassinosteroid Insensitive 1-Associated Receptor Kinase	BRIK
Carotenoid Cleavage Dioxygenase 8	CCD8
Constans	CO
Cryptochrome 1	CRY1
Cup Shaped Cotyledon 3	CUC3
Cycling Dof Factor	CDF
Cytokinin	CK
Dawdle	DDL
DICER-like	DCL
Differentially Expressed	DE
dsRNA-Binding Protein 4	DRB4
Enhancer of Zeste 2	E(z)2
Flavin-Binding Kelch Repeat F-Box Protein 1	FKF1
Florigen activation complex	FAC
FLOWERING LOCUS C	FLC
FLOWERING LOCUS T	FT
GA related MYB	GAMYB
Gene Ontology	GO
Gene Regulatory Module	GRM
Geranylgeranyl Diphosphate	GGPP
Gibberellins	GA
GIGANTIA	GI
Homeodomain Leucine Zipper Protein III	HD-ZIP III
Hyponastic Leaves 1	HYL1

Identity of Tuber 1	IT1
Indole Acetic Acid	IAA
Internal Standard	IS
Jasmonic Acid	SA
Knock-down	KD
Leafbladeless 1	LBL1
Lipoxygenase	POTLX1
Long day	LD
Meiosis Arrested At Leptotene 1	MEL1
Messenger RNA	mRNA
Multicopy Suppressor of IRA 1	MSI1
NAM, ATAF, and CUC Transcription factor	NAC
Nucleotide-Binding Domain Leucine-Rich Repeat Proteins	NB-LRR proteins
Outer Cell Layers 4	OCL4
Overexpression	OE
Pentatricopeptide Repeat	PPR
Phased siRNAs	phasiRNAs
Phytochrome A	PHYA
Phytochrome B	PHYB
Phytochrome F	PHYF
Pinhead 4	PIN4
Polypyrimidine Tract-Binding 1	PTB1
Post-Transcriptional Gene silencing	PTGS
Potato Homeobox 1	POTH1
Related to Apetella2 1	RAP1
RNA Dependent RNA Polymerase 6	RDR6
RNA Induced Silencing Complex	RISC
RNA Ligase-Mediated Rapid Amplification of Complementary DNA Ends	RLM-RACE
Salicylic Acid	SA
Self-Pruning 6A	SP6A
Serrate	SE
Shoot Apical Meristem	SAM
Short Day	SD

Short Target Tandem Mimic	STTM
Short-Root	SHR
Solid Phase Extraction	SPE
Squamosa Promoter Binding-Like	SPL
Standard error of Means	SEM
Stolon apical meristem	STAM
Strigolactone	SL
Sucrose Transporter 4	SUT4
Suppressing Expression of SP6A	SES
Suppressor Of Gene Silencing 3	SGS3
Teosinte branched, cycloidea (cyc) and PCF	TCP
Three Amino Loop Extension	TALE
Tomato Lonely Guy 1	TLOG1
ToMV Resistance Protein	Tm2
Trans-Acting Short Interfering RNAs	TasiRNA
Transcription Factor	TF
Transport Inhibitor Response 1	TIR1
Tryptophan Aminotransferase Of Arabidopsis (TAA)/YUCCA	YUCCA
Tuberigen Activation Complex	TAC
Vector Control	VC
Wild Type	WT

Synopsis

Investigating the functions of small non-coding RNAs in stolon-to-tuber transition stages of potato development

Name: Nilam Namdeo Malankar

Reg. No: 20142002

Name of Supervisor: Prof. Anjan K. Banerjee.

Department: Biology

Date of Registration: 01st August 2014

Indian Institute of Science Education and Research, Pune, India.

Introduction

Stolon-to-tuber transition is a crucial part of potato development. This process is controlled by photoperiod and is well coordinated *via* the action of phytohormones and transcriptional factors (Hannapel et al., 2017; Kondhare et al., 2020). In this process, a belowground modified stem (stolon) undergoes developmental transitions and accumulates starch to form a tuber (Xu et al., 1998b). Given a large number of studies focused on the critical regulators of potato development (tuberization), their precise roles in governing stolon-to-tuber transitions is less explored. A decade-long work in this direction has uncovered hormonal networks regulating tuberization. Reduced levels of gibberellin and higher auxin at early stolon stage are essential for this transition (Roumeliotis et al., 2012b). This is achieved by the controlled expression of their metabolism genes (Kloosterman et al., 2007; Carrera et al., 2000; Bou-torrent et al., 2011; Roumeliotis et al., 2013). However, how plants maintain this hormonal balance throughout the developmental stages remains unknown. Small non-coding RNAs have been shown to regulate various biological processes in plants and animals (Liu et al., 2020; Dong et al., 2022). Especially, miRNAs participate in nearly all physiological events, such as shoot apical meristem (SAM) development and maintenance, leaf morphogenesis, juvenile-to-adult phase transition, flowering and root development (Dong et al., 2022). Another subset of small RNAs, known as phasiRNAs, shown to have a role in plant growth and stress responses. Functions of phasiRNAs in plant development have been well described in *Arabidopsis*, maize and rice (Liu et al., 2020). Small RNAs regulate hormonal networks by controlling target genes. A recent study in wheat showed that miR9678, a wheat-specific miRNA, targets long non-coding RNA (lncRNA), WSGAR, to produce phasiRNAs that regulate the germination process *via* controlling the expression of GA metabolism genes. Absciscic acid (ABA) signaling proteins regulate miR9678 expression, which modulates GA response in seeds (Guo et al., 2018). These

results suggest that small RNAs can orchestrate hormonal networks to fine-tune developmental processes.

A few miRNAs, such as miR172, miR156 and miR390 have been demonstrated to regulate tuber development. miR172 acts as a positive regulator of tuberization (Martin et al., 2009) and miR156 regulates miR172 expression. Interestingly, overexpression of miR156 showed profuse stem branching and aerial stolons that formed tubers under inductive conditions by regulating cytokinin biosynthesis in the axillary meristems (Bhogale et al., 2014). Recently, Lehretz et al. (2019) demonstrated the role of a small RNA, SES that targets *SP6A* mRNA to inhibit tuberization at high temperatures. These reports indicate that small RNAs can govern the tuber developmental process. However, their exact role in stolon-to-tuber transitions have not been investigated. Previous studies have profiled miRNA populations from potato (Yang et al., 2009; Xie et al., 2011; Zhang et al., 2013), however, their functional importance in tuber development is not studied. Lakhotia et al. (2014) identified several conserved and potato-specific miRNAs from early stolon stages and revealed unique small RNAs that might be potentially involved in stolon transitions in potato. However, this study was carried out independent of photoperiod effect. As day-length is a crucial inducer for tuberization (Ewing and Struik, 1992; Rodriguez-Falcon et al., 2006), we speculate that photoperiod might affect the functions of small RNAs during stolon-to-tuber transitions. Therefore, we conducted small RNA sequencing of early stolon developmental stages under differential photoperiod (SD-inductive vs LD-non-inductive) conditions. Knowledge of unique small RNA populations involved in stolon-to-tuber transitions will enable us to identify crucial regulators that might govern hormonal networks during stolon-to-tuber transition stages of potato. Hence, using *Solanum tuberosum* ssp. *andigena* as model system, we designed following objectives for this study.

1. To identify small RNAs from early tuber transition stages of potato under differential (LD vs SD) photoperiodic conditions.
2. To decipher the role of novel phasiRNA [siRD29(-)] in stolon-to-tuber transition stages.
3. Investigating the function of a novel potato-specific miRNA (miRNA72) during potato development.

1. To identify small RNAs from early tuber transition stages of potato under differential photoperiodic conditions

Tuber development in potato typically requires a time window of 25-28 days after switching the plant to tuber-inductive conditions. During this time window, hooked stolon (0-4 days) opens up due to initiation of swelling at the base of the hook (~7-8 days). Further, tissue swelling expands to form swollen stolons (10-14 days). To determine the small RNAs potentially involved in the early stages of stolon-to-tuber transitions (4, 7 and 10 days) in a photoperiod-dependent manner, we carried out a deep-sequencing using stolon samples of a photoperiod-sensitive potato cultivar (*S. tuberosum* ssp. *andigena*) grown under differential photoperiodic conditions (Long-day versus Short-day). We have identified 7 (out of 324) conserved and 12 (out of 311) novel miRNAs, differentially expressed in stolon stages under SD versus LD photoperiod. Two conserved miRNAs, such as Stu-miR479 and Stu-miR319b, with their confirmed targets *StGRAS* and *StGAMYB* showed inverse expression patterns across the stolon stages, suggesting their potential role in tuber development. We also identified several potato-specific miRNAs that might target tuberization related genes and epigenetic factors. Further, our *in silico* analyses revealed that phasiRNAs could be involved in tuber development. Altogether, 24 *TAS-like* loci (out of 830) were found to be triggered by miRNAs identified in our study. We have predicted targets for 190 phased siRNAs from 24 *TAS-like* loci that includes key tuberization genes, such as *StCONSTANS*, *StCDF1*, *StFT1*, *StSP5G*, *StBEL5*, GA-auxin related genes, sucrose synthesis and transporters etc. Overall, our study indicated that sRNAs and their selective target genes could be associated with the regulation of early stolon stages. Our goal is to delineate candidate small RNAs that might regulate GA and auxin hormonal balance, the two major hormones involved during stolon-to-tuber transitions. Interestingly, we noticed that one of the phasiRNA, siRD29(-) could target a GA biosynthesis gene, *StGA3ox3*, in potato. This led us to hypothesize that the siRD29-*StGA3ox3* gene regulatory module may be involved in fine-tuning the hormonal (or GA) balance in stolon-to-tuber transition stages.

2. To decipher the role of a novel phasiRNA [siRD29(-)] in stolon-to-tuber transition stages

From our small RNA sequencing data sets, we identified two putative *TAS-like* loci located in a *disease resistance* gene, *StRGA4*. We also demonstrated that a miR482 family member, Stu-miR482c cleaves a *StRGA4* transcript in potato and generates phasiRNAs. Reduced GA levels is a pre-requisite for stolon to tuber transition. Previous studies have reported that this reduction of GA level is a cumulative effect of a decreased expression of GA biosynthesis genes (*StGA20ox1* and *StGA3ox2*) (Carrera et al., 2000; Bou-torrent et al., 2011)

and an increase in GA catabolism genes (*StGA2ox1*; Kloosterman et al., 2007). However, the function of this *StGA3ox3* isoform remains unknown in potato. In this study, we have demonstrated that Stu-miR482c could trigger siRD29(-) generation from *StRGA4* transcript using a transient expression system in *Nicotiana benthamina*. Using quantitative PCRs and small RNA sequencing from co-infiltrated leaves, we proved that siRD29(-) is generated at the 29th position from the antisense strand of *StRGA4* transcript in a RDR6-dependent manner and it cleaves *StGA3ox3* transcript *in planta*. SiRD29(-) and *StGA3ox3* showed the inverse correlation of expressions in SD-induced stolons, indicating a possible regulation of *StGA3ox3* mRNA in stolons. *StGA3ox3* overexpression and antisense showed GA-mediated phenotypes supporting its function as a GA biosynthesis gene in potato. Further, we observed that *StGA3ox3*-AS (antisense) and *StRGA4*-OE (overexpression) lines had higher tuber numbers than WT potato plants probably due to altered GA-auxin levels. Notably, *StRGA4*-OE lines exhibited increased levels of siRD29(-), followed by a decrease in *StGA3ox3* transcripts in leaves as well as stolons similar to *StGA3ox3*-AS lines. These lines also showed decreased levels of bioactive GA₃ in shoot apices. Overall, these findings establish that the Stu-miR482c-*StRGA4*-siRD29(-)-*StGA3ox3* gene regulatory module (GRM) is functional in stolons. Further, through promoter activity and infiltration assays, we have showed that StBEL5 (a positive regulator of tuberization) induces *StGA3ox3* expression in the potato plant. Finally, we put forward a model with a possible dual regulation of *StGA3ox3* in stolons by two independent regulatory pathways: (i) through siRD29(-) and the other (ii) is mediated by StBEL5. Overall, our findings suggest that how plants possibly employ a dual gene regulatory mechanism to maintain a minimal bioactive GA level during tuber development by controlling the expression of the GA biosynthesis gene, *StGA3ox3*.

3. Exploring the function of a potato-specific miRNA (miR72) during potato development

Previously, a small RNA sequencing study (Lakhotia et al., 2014) reported 147 potato specific miRNAs in the Indian cultivar Kufri Chandramukhi. From the datasets, based on the miRNA abundance, putative tuberization related target genes, and differential expression from stolon-to-tuber transition stages, we selected candidate potato specific miRNAs, miR34, miR53, miR72 and miR111 for further analysis. Interestingly, we noted that miR72 levels reduced significantly under tuber-inducing short-day conditions, suggesting its potential role in tuber development. *Cryptochrome 1b* and TCP transcription factor (*StBRC1b/StIT1*) were found to be the putative targets of miR72. *StIT1* (a key tuber identity gene) shown to interact with StSP6A (positive regulator of tuberization) and StIT1-SP6A complex was found to be

necessary for tuberization (Tang et al., 2022). Based on the reduced expression of miR72 across the stolon transition stages and the necessity of *StIT1* gene in tuber development, we speculate that miR72-*StIT1* (miRNA-target pair) may be involved in tuberization. We have generated miR72-OE and STTM72-KD lines for the functional characterization of miRNA72 and demonstrate that miR72-OE lines produce sessile tuber phenotype. These plants also exhibited reduced tuber yield and root biomass. We noticed that STTM72-KD lines did not show sessile tubers and tuber yield was not different than the wild type. Further, sessile tuber formation phenomenon was found to be independent of day-length as miR72-OE Désirée lines (a day-neutral potato cultivar) also showed sessile tubers. Through the cleavage site mapping assay, *Cryptochrome 1b* gene was confirmed to be a target gene of miR72. We also observed that *StIT1* gene expression is reduced in miR72-OE line, suggesting that *StIT1* might be targeted by an increased level of miR72 in the OE line. Overall, our results suggest that miR72 could be involved in defining early stages of stolon development. Only future experiments can further delineate the key role of miR72 in this process.

Summary

To understand the role of small RNAs in stolon-to-tuber transition stages, we have carried out small RNA sequencing of stolons developed under photoperiodic conditions (SD and LD) and identified many differentially expressed miRNAs and phasiRNAs, which were found to potentially target the key tuberization pathway genes. Our primary focus was to investigate the role of small RNAs that may be governing the key genes involved in the regulation of gibberellin and auxin level during stolon-to-tuber transitions. We showed that a novel phasiRNA, siRD29(-) targets a key GA biosynthesis gene -*StGA3ox3* in potato. Through multiple experimental evidences, we demonstrate that a gene regulatory module (GRM), Stu-miR482c-*StRGA4*-siRD29(-)-*StGA3ox3* is functional in potato development. Finally, we have presented a model for dual regulation of *StGA3ox3* gene in stolon-to-tuber transitions to maintain minimal gibberellin levels in stolons for fine-tuning the tuber transition events. Additionally, we have attempted to identify the function of a highly abundant potato specific miRNA (miR72) during tuber development. We showed that miR72 could be involved in early stages of stolon development. This study has unraveled the role of novel small RNA regulatory networks governing the stolon-to-tuber developmental process in potato.

Reference

- Bhogale S, Mahajan AS, Natarajan B, Rajabhoj M, Thulasiram HV, Banerjee AK** (2013). MicroRNA156: A Potential Graft-Transmissible MicroRNA That Modulates Plant Architecture and Tuberization in *Solanum tuberosum* ssp. *Andigena*. *Plant Physiol* 164, 1011–1027
- Bou-Torrent J, Martínez-García JF, García-Martínez, JL, Prat S** (2011). Gibberellin A1 metabolism contributes to the control of photoperiod-mediated tuberization in potato. *PLoS One* 6(9): e24458
- Carrera E, Bou J, García-Martínez JL, Prat S** (2000) Changes in GA20-oxidase gene expression strongly affect stem length, tuber induction and tuber yield of potato plants. *Plant J* 22(3): 247-256
- Chitwood, D. H., Nogueira, F. T., Howell, M. D., Montgomery, T. A., Carrington, J. C., & Timmermans, M. C.** (2009). Pattern formation via small RNA mobility. *Genes & development*, 23(5), 549-554.
- Dong Q, Hu B, Zhang C** (2022) microRNAs and their roles in plant development. *Front Plant Sci* 13
- Ewing, E. E., & Struik, P. C.** (1992). Tuber formation in potato: induction, initiation, and growth. *Horticultural reviews*, 14, 89-198.
- Fei Q, Xia R, Meyers BC** (2013) Phased, secondary, small interfering RNAs in posttranscriptional regulatory networks. *Plant Cell* 25 (7): 2400-2415
- Guo G, Liu X, Sun F, Cao J, Huo N, Wuda B, Yao Y** (2018) Wheat miR9678 affects seed germination by generating phased siRNAs and modulating abscisic acid/gibberellin signaling. *Plant Cell* 30(4): 796-814
- Hannapel DJ, Sharma P, Lin T, Banerjee AK** (2017) The multiple signals that control tuber formation. *Plant Physiol* 174(2):845-856
- Kloosterman B, Navarro C, Bijsterbosch G, Lange T, Prat S, Visser RGF, Bachem CWB** (2007) StGA2ox1 is induced prior to stolon swelling and controls GA levels during potato tuber development. *Plant J* 52: 362-373
- Knauer, S., Holt, A. L., Rubio-Somoza, I., Tucker, E. J., and Hinze, A.** (2013). A protodermal miR394 signal defines a region of stem cell competence in the Arabidopsis shoot meristem. *Dev. Cell* 24, 125–132. doi: 10.1016/j.devcel.2012.12.009
- Kondhare KR, Natarajan B, Banerjee AK** (2020) Molecular signals that govern tuber development in potato. *Int J Dev Biol* 64(1-2-3): 133-140.
- Lakhotia N, Joshi G, Bhardwaj AR, Katiyar-Agarwal S, Agarwal M, Jagannath A, Goel S, Kumar A** (2014) Identification and characterization of miRNAome in root, stem, leaf and tuber developmental stages of potato (*Solanum tuberosum* L.) by high-throughput sequencing. *BMC plant biology* 14: 6

- Lehretz, G. G., Sonnewald, S., Hornyik, C., Corral, J. M., & Sonnewald, U.** (2019). Post-transcriptional regulation of *FLOWERING LOCUS T* modulates heat-dependent source-sink development in potato. *Current Biology*, 29(10), 1614-1624.
- Liu Y, Teng C, Xia R, Meyers BC** (2020) PhasiRNAs in plants: Their biogenesis, genic sources, and roles in stress responses, development, and reproduction. *Plant Cell*, 32(10): 3059–3080
- Martin, A., Adam, H., Díaz-Mendoza, M., Zurczak, M., Gonzalez-Schain, N. D., & Suarez-Lopez, P.** (2009). Graft-transmissible induction of potato tuberization by the microRNA miR172. *Development*, 136(17), 2873-2881.
- Potato Genome Sequencing Consortium** (2011) Genome sequence and analysis of the tuber crop potato. *Nature* 475:189–195
- Rodríguez-Falcón, M., Bou, J., & Prat, S.** (2006). Seasonal control of tuberization in potato: conserved elements with the flowering response. *Annu. Rev. Plant Biol.*, 57, 151-180.
- Roumeliotis E, Visser RG, Bachem CW** (2012b) A crosstalk of auxin and GA during tuber development. *Plant Signal Behav* 7(10): 1360-1363
- Roumeliotis E, Kloosterman B, Oortwijn M, Lange T, Visser RG, Bachem CW** (2013) Down regulation of StGA3ox genes in potato results in altered GA content and affect plant and tuber growth characteristics. *J Plant Physiol* 170(14): 1228-1234
- Xie F, Frazier TP, Zhang B** (2011) Identification, characterization and expression analysis of MicroRNAs and their targets in the potato (*Solanum tuberosum*). *Gene* 473: 8–22
- Xu X, Vreugdenhil D, van Lammeren AAM** (1998b) Cell division and cell enlargement during potato tuber formation. *J Exp Bot* 49: 573-582
- Yang, T., Wang, Y., Teotia, S., Wang, Z., Shi, C., Sun, H., & Tang, G.** (2019). The interaction between miR160 and miR165/166 in the control of leaf development and drought tolerance in *Arabidopsis*. *Scientific Reports*, 9(1), 2832.
- Zhang R, Marshall D, Bryan GJ, Hornyik C** (2013) Identification and characterization of miRNA transcriptome in potato by high-throughput sequencing. *PloS one* 8: e57233
- Zhang W, Luo Y, Gong X, Zeng W, Li S** (2009) Computational identification of 48 potato microRNAs and their targets. *Computational Biology and Chemistry* 33: 84–93

Chapter 1

Introduction

1.1 Potato

Potato is the fourth most important food crop in the world right after Rice, Wheat and Maize (<https://education.nationalgeographic.org/resource/wbt-staple-food-crops-world/>). It grows as herbaceous perennial crop and belongs to *Solanaceae* family. Other members of this family such as Tomato, Eggplant and Bell pepper are also used as vegetable food for human consumption. Potatoes are native to Andes region in South America. Therefore, potato varieties originated from this region are often dependent on day-length for potato formation and tuberize under short day (SD) conditions (Rodríguez-Falcón et al., 2006). On contrary, cultivated potato varieties are usually not affected by day-length hence termed as day-neutral varieties. Potato plants produce berries like fruits with many seeds. However, potatoes are cultivated for their tubers (Potato tuber) which are belowground modified stems loaded with starch and other nutrients. Tubers served as storage organs and a mode of asexual reproduction. Under favorable conditions, sprouts arise from the dormant nodes (eyes) of the tuber giving rise to new plantlets under light conditions. Agro-economic importance of potato tubers is because of its nutritional value. They are rich in carbohydrates, proteins, fibers and vitamins and are able to supplement dietary needs. Hence, understanding the process of potato development is of great importance for future food security purposes.

1.2 Tuberization

Tuberization or tuber developmental pathway is a complex process wherein a stolon originates from the main axis of the plant below soil and under tuber inductive conditions, it undergoes transitions to form mature tuber or potato (Sarkar, 2010). The entire process is under the influence of various environmental, hormonal and molecular factors, as described in the subsequent sections (Hannapel et al., 2017; Kondhare et al., 2021). Tuberization comprises of three phases: tuber induction, initiation and proliferation. In past, researchers thought that, the stimulus for tuber induction is generated in the leaf clusters influenced by light. However, the signal for tuberization remained elusive until a grafting experiment between flowering tobacco (scion) and not-tuberizing potato (stock) was conducted in 1981 (Chailakhyan et al., 1981). This grafting resulted in the induction of tuber formation in potatoes suggesting the presence of common signals between flowering and tuberization processes. This also indicated the possibility of mobile signals regulating these pathways which is described in this chapter. From the past several years, scientists have been investigating tuberization pathway in order to enhance tuber quality and yield. Potato genome was made available in 2011, which accelerated

the research for functional analysis of genes involved in tuberization (“Genome Sequence and Analysis of the Tuber Crop Potato,” 2011). The following sections describe molecular details of tuber development process.

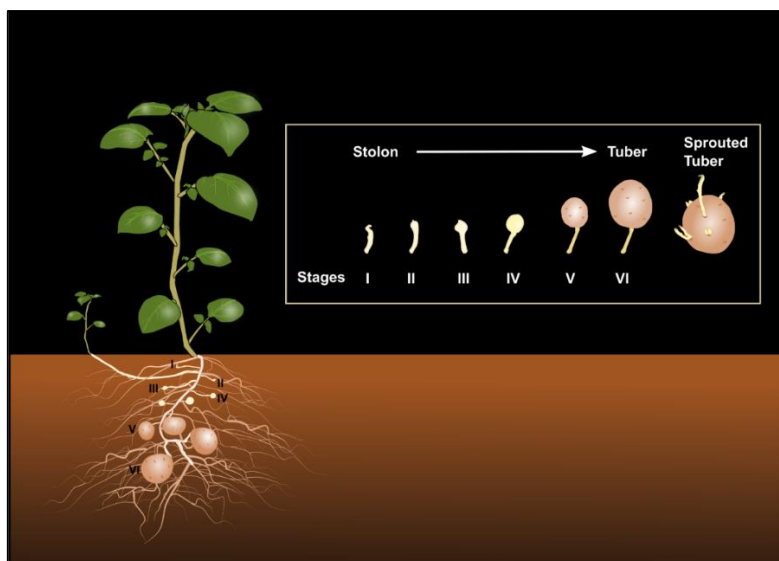


Fig. 1.1 Pictorial representation of potato plant showing stolon-to-tuber transitions: belowground modified stem (stolon) passes through transition stages (I-VI) to form mature tuber. Stages, I-stolon; II and III- swollen stolon; IV-minituber; V and VI- Mature tuber. (Adapted from Kondhare et al., 2020)

1.2.1 Stolon-to-tuber transitions

Under tuber inductive conditions, the stolon undergoes developmental transitions before it matures to the tuber (Fig. 1.1). These stages comprise of stolon, swollen stolon, minituber, and mature tuber (Xu et al., 1998). The process of tuber formation is completed over a period of 25 days, thereafter, the tuber matures by accumulating starch to a greater extent (Kloosterman et al., 2005) (Fig. 1.2). At the onset of tuberization, the growth of stolon apical meristem (STAM) arrested, and cell division ceases. Initially, cells from the sub-apical region of stolons exhibits growth by transverse cell division resulting in stolon elongation. Under inductive conditions, the pattern of cell division from sub-apical region changes from transverse to longitudinal, resulting in radial expansion of stolon, to form swollen stolon. This cell division and expansion occur within the cortex and pith of the tuber (Xu et al., 1998). Critical hormone levels in stolons direct the stolon-to-tuber transition phenomenon. Notably, rapid decrease in Gibberellin (GA) levels in stolon post induction causes re-orientation of microtubules in cells from subapical region, leading to a change in cell division pattern (Roumeliotis et al., 2012). Many tuberization related studies focused on factors affecting overall tuber development. Kloosterman et al. (2005) attempted to study differentially

expressed genes in eight tuber developmental stages with the help of cDNA microarrays, and reported expression profiles of several genes involved in the process (Kloosterman et al., 2005). Potato genome was published much later in 2011 (“Genome Sequence and Analysis of the Tuber Crop Potato,” 2011). With the help of transcriptomic approaches, a detailed analysis of factors governing stolon transition stages can help in understanding the dynamics of stolon-to-tuber transitions.

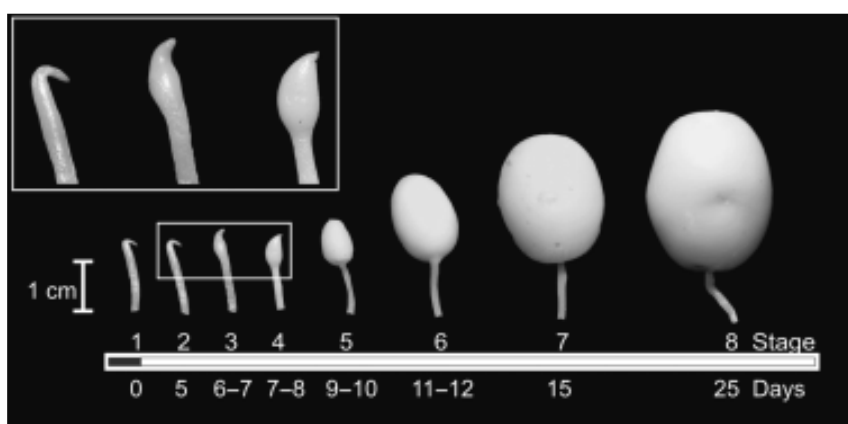


Fig. 1.2 Timeline for tuber developmental stages of potato. Tuber transition stages observed over a period of 25 days in potato, after switching to Short-day (SD) conditions. Stages 1- Long day (LD) stolon, 2- SD stolon, stages 3 and 4- swollen stolon; 5- mini-tuber stage. Numbers below the white line indicates days after SD induction (DAS) when the stages were noticed. (Adapted from Kloosterman et al., 2005)

1.2.2 Environmental Factors that influence tuber formation

Tuber development process is under the influence of environmental factors such as photoperiod, temperature and nitrogen levels etc. The role of these factors are described in the following sections.

1.2.2.1 Role of photoperiod

Photoperiod is one of the major exogenous factor that influences several plant developmental processes. Photoperiod defines as length of day and night in a 24 hours cycle. Short day (SD; 8hours of light and 16 hours of dark) are found to be tuber inductive while long days (LD; 8hours of light and 16 hours of dark) are tuber repressive (Jackson, 2009) for potato plants. Flowering and tuberization are greatly affected by photoperiod (Ewing & Struik, 1992). Arabidopsis flowers in LD conditions but fails to do so under SD (Jackson, 2009) condition. Andean varieties of potato tuberizes under harsh winter conditions. Where tuber remain dormant throughout winter and sprouts in favorable growth conditions. Thus, these varieties

have adapted strict SD conditions for tuberization, whereas mostly cultivated potato varieties adapted to day neutral conditions (Rodríguez-Falcón et al., 2006). 8 hours (SD) of light promotes tuber formation in SD specific plants, but do not form tubers under LD or SD with night break (NB) (Jackson, 2009).

Grafting experiment in potato plants with SD induced scion on LD grown stock, resulted in tuber formation in non-induced stock potato plants (Gregory, 1956). This suggested that SD photoperiod induces expression of tuberization signal that is graft-transmissible causing tuber formation in non-induced potato plants (KUMAR & WAREING, 1974). Few years later, phytochromes such as PHYB and PHYF were demonstrated to sense light signals and relay the information to the tuber inducing mobile signals.

1.2.2.2 Role of temperature

The process of tuberization is triggered by lower temperatures and suppressed at elevated temperatures (Werner, 1934; Gregory, 1956). As mentioned earlier, potatoes originated from South America and they produced tubers to deal with harsh weather and cooler temperatures. Hence, it is observed that potatoes tuberize at cooler temperatures such as 18-20 °C, and constant high temperatures such as 28-32 °C, inhibit this process. A study led by Werner (1934), suggested that temperature and photoperiod can be correlated. Higher temperature can be associated to longer days and during short day, day-length is less and plant experiences low temperature conditions. This study indicated that temperature and photoperiod could converge at some point in tuberization pathway (Werner, 1934). Recent advances in potato related research have shed lights on temperature dependent molecular mechanism of tuber development. For example, high temperatures have shown to inhibit a FLOWERING LOCUS T (StSP6A) protein, positive regulator of tuberization via induction of small RNAs (Lehretz et al., 2019). This mechanism is described in section 1.5.4

1.2.2.3 Effect of Nitrogen levels

Nitrogen content affects tuber development. Low nitrogen supply promotes tuberization whereas, nitrogen rich environment inhibits tuber formation (Werner, 1934). Excessive nitrogen causes increase in gibberellin content of stolons leading to increase stolon length and rate of tuberization. Further it affects amino acids and starch content of tubers.

1.3 Hormonal regulation of tuber formation

Tuber development is under the control of inter-connecting phytohormone pathways. Several hormones such as gibberellins (GA), auxins, cytokinins (CK), strigolactones, jasmonic acids (JA) and abscisic acids (ABA) influence tuberization process which are described in the following sections.

1.3.1 Gibberellin (GA)

Gibberellic acid (GA) exerts a control over plant growth and differentiation by tightly regulating its concentration (Rodríguez-Falcón et al., 2006; Sarkar, 2010). The role of GAs during stolon-to-tuber transitions is well investigated. Application of bioactive GA₃ inhibited tuber formation, while GA biosynthesis inhibitors such as paclobutrazol enhanced tuber formation (Xu et al., 1998). These results suggest that GA inhibits tuberization. A study by Carrera et al., 2000, manipulated GA₂₀ and GA₁ levels by overexpression and downregulation of *StGA20ox1* gene. *StGA20ox1-OE* lines resulted in delayed tuber formation under short-day conditions. Both lines did not tuberize under LD conditions, indicating GA couldn't overcome the effect of photoperiod (Carrera et al., 2000).

It was believed that, gibberellins pose an inhibitory effect on tuber development, and thus bioactive GA levels should be minimal in plants. Microarray analysis from SD induced stolons showed increased *StGA2ox1* (a GA catabolic enzyme) transcripts in tuber forming stolons (Kloosterman et al., 2005). Upregulation of *StGA2ox1* ensures minimum GA concentration in stolons, to ensure tuber formation. Another study from the Salomé's group in 2011, identified GA biosynthesis genes *StGA3ox1* and *StGA3ox2* in potato. Of which, *StGA3ox2* identified as rate limiting enzyme and catalyzes GA₁ production in stolons (Bou-Torrent et al., 2011). Constitutive overexpression lines of *StGA3ox2* exhibited taller phenotype and showed a delayed tuber formation, whereas leaf-specific overexpression lines (*LS1:StGA3ox2*) tuberizes earlier than wild type, contradicting to the widely accepted notion of GA inhibiting tuberization (Bou-Torrent et al., 2011). This phenomenon was explained based on differential mobility of GA precursors throughout the plant. In *LS1:StGA3ox2* lines, GA₂₀ precursor in aerial tissue utilized by *StGA3ox2* to generate GA₁ form, reducing the availability of GA₂₀ precursor for transporting to stolons. Due to substrate decrease, the GA 3-oxidase activity in the stolons produces less amount of bioactive GA₁ and plant showed early tuberization phenotype. Roumeliotis et al. (2013), generated RNAi lines of *StGA3ox2* producing plants with smaller height but no change in time for tuberization. Instead, these lines

produced more number of tubers per plant compared to WT (Roumeliotis et al., 2013). Authors suggested that this phenotype might be due to altered GA:auxin ratio in stolons (Roumeliotis et al., 2012). Altogether, feedback regulation of gibberellin levels in stolons is crucial for proper tuber development.

1.3.2 Auxin

Auxins play a key role in regulating different aspects of plant architecture such as embryogenesis, organogenesis and lateral root formation etc. (Teale et al., 2006). They also have significant function during stolon-to-tuber transitions. Auxin application to the *in vitro* single nodal potato explants resulted in early tuberization with sessile tubers (Xu et al., 1998), suggesting its putative role in tuber formation. Kloosterman and group (2008) performed microarray for stolon developmental stages and identified differential expression of auxin related genes. *ARF6* (Auxin response factor 6) expression increases in swollen stolons (Kloosterman et al., 2008). Auxin levels increase at the onset of tuberization coinciding with the increased expression of IAA biosynthesis genes (*StYUC-like1*) and auxin transporters (*PIN* family members) (Roumeliotis et al., 2013). Recently, the same group demonstrated that constitutive expression of *StYUC-like1* (*StYUCCA-8*) resulted in altered auxin content with increased tuber numbers but tuber size is reduced (Roumeliotis et al., 2023). Authors have correlated the increased auxin content with altered shoot and stolon branching, suggesting that the regulation of auxin content is also crucial for tuber formation.

1.3.3 Cytokinin

Cytokinin (CK) also plays an important role during tuber formation. Application of CK to *in vitro* nodal explants promoted tuberization. However, it failed to replicate results in soil-grown plants (PALMER & SMITH., 1970). CK levels were increased in tuber inducing stolon tips. But CK alone could not induce tuberization (Mauk & Langille., 1978). These results suggest that cytokinin is not necessary for tuber induction but it involves in tuber developmental stages. Eviatar-Ribak and co-workers (2013) expressed *TLOG1* (*Tomato Lonely Guy1*), a CK biosynthesis gene in tomato plants. Increased *TLOG1* levels resulted in aerial tuber production from the axillary meristems of tomato plant which, usually do not produce any tubers (Eviatar-Ribak et al., 2013). Cytokinin biosynthesis genes are shown to be under control of polycomb repressor proteins such as *MSI1* and *BMI1* (Kumar et al., 2020). Overall, these studies highlighted the significant role of CK in tuberization.

1.3.4 Strigolactones

Strigolactones (SLs) work together with auxins but, have an inhibitory effect on shoot branching. *In vitro* application of SLs to nodal explants resulted in inhibition of axillary bud outgrowth and subsequent production of tubers (Roumeliotis et al., 2012). SLs have not been detected in stolons yet. However, SLs have been detected in root extracts of potato plants (López-Ráez et al., 2008). *CAROTENOID CLEAVAGE DIOXYGENASE 8 (CCD8)* is a key gene in SL biosynthesis pathway (Alder et al., 2012). *CCD8* RNAi lines of potato have shown reduced stolon formation and dwarfing phenotype in plants (Pasare et al., 2013). Based on the results, it is conceivable that strigolactones have potential role during tuberization along with auxin. However, the detailed mechanism has not been well investigated.

1.3.5 Jasmonic acid

Jasmonic acid (JA) are known to regulate plant physiological responses to abiotic and biotic stresses and it does so by inducing a series of gene expressions. Pelacho and Castel (1991) treated *in vitro* nodal cuttings of potato plants with different concentrations of JA, and found out that JA induces tuber formation in stolons with an optimum concentration. Beyond the threshold, its effect reduces (Pelacho & Mingo-Castel., 1991). However, no further studies were carried out about *in vivo* effect of JA on tuberization. Castro et al., (1999) showed that JA induces cell expansions in tuber buds. They also demonstrated that JA can act on young cells and induces radial expansion of meristematic cells indicating that JA might have a role in stolon transition stages (Takahashi et al., 1994).

1.3.6 Absciscic acid

Absciscic acid (ABA) has tuber promoting effect in potato. Application of ABA to potato plants in soil, resulted in early tuberization phenotype and higher tuber numbers. In presence of ABA, nodal explants tuberized earlier than control ones indicating that ABA induces tuber formation in presence of high sugar and GA (Xu et al., 1998). However, ABA deficient lines of *Solanum phureja* tuberizes similar to wild-type. Authors concluded that ABA is not required for tuberization process, but it can promote one (Quarrie, 1982). ABA works antagonistically with GA in plants. Therefore, it promotes tuberization due to its antagonistic relationship with GA.

1.3.7 Gibberellin (GA) and auxin cross talk during tuber development.

Plant growth and development is controlled by mutual interactions of plant hormones. As described earlier various phytohormones have been shown to regulate various aspects for tuberization pathway and their action can be integrated into one ultimate effect of stolon-to-tuber transition. Of these, GA and auxin have been demonstrated to work in concerted manner to regulate tuber development (Roumeliotis et al., 2012).

Under non-inductive LD conditions, high GA levels causes elongation of stolons (stolon growth phase) due to cell division and expansions. Auxin levels remain low, helping plant to maintain the growth of stolon apical meristem (STAM) (Roumeliotis, Kloosterman, et al., 2012). At the onset of tuberization, mobile signal transports from leaf to stolons and activate expression of various auxin and GA related genes. Subsequently, the GA level reduces and auxin levels rise due to increased expression of auxin biosynthesis genes (Roumeliotis et al., 2012). It causes a change in the cell division plane of the sub-apical region in stolons. This results in reduction of longitudinal stolon growth and radial expansion of tissue begins, signifying transition of stolon to swollen stolon. Reduction in GA content is a key step for this developmental event. Auxin levels rapidly increases at the initial stolon stages and once swollen stolon starts maturing, its level decreases (Fig. 1.3). Continual supply of auxin to stolon tips during stolon-to-tuber transitions inhibited tuber formation (Roumeliotis, Kloosterman, et al., 2012). This suggests that auxin maxima is needed for the cell division switch occurring after tuber induction. Molecular mechanism behind the auxin driven cell division during the tuberization is not clearly understood. Further research may shed lights on the mode of action.

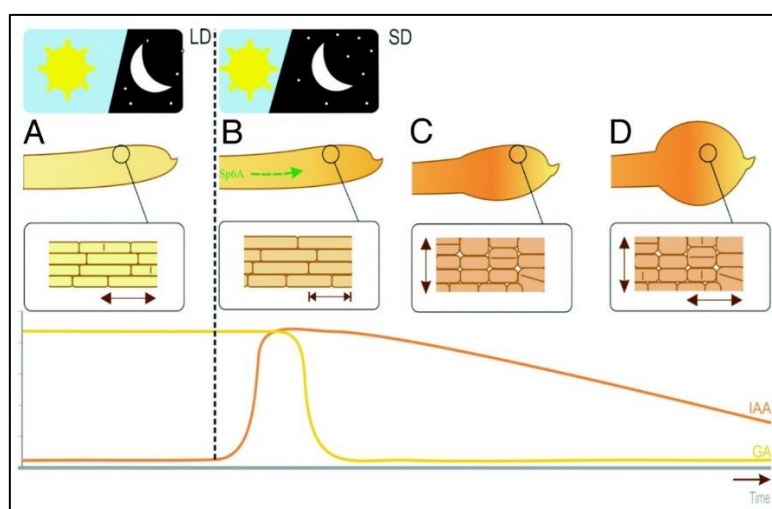


Fig 1.3 GA and IAA regulation at stolon tips during stolon-to-tuber transitions. (A) GA content is initially high in stolon under inhibitory LD conditions (B) SP6A protein moves from leaves to stolons (green arrow) under tuber inducing conditions. Reduction in GA accompanied by increase in IAA levels in stolon tips is observed. (C) Changes in the plane of cell division resulting in radial expansion of stolons. (D) Accumulation of starch in stolons to form mature tuber. (Adapted from Roumeliotis et al., 2012)

1.4 Molecular mechanism of photoperiod dependent tuberization

Flowering and tuberization share similar cues in their signaling pathway. Tuberization is strictly SD dependent phenomenon in andigena, such that this plant does not tuberize under LD conditions. Photoperiod dependent light signals are perceived by photoreceptors such as phytochromes and cryptochromes. Jackson and co-workers demonstrated the role of phytochrome, *PHYB* in tuberization. They show that *PHYB* antisense lines of andigena were photoperiod independent and tuberized under non-inductive LD conditions stating its importance (Jackson et al., 1998). Another phytochrome *PHYF*, was demonstrated to regulate tuberization possibly by forming heterodimer with *PHYB*. Similar to *PHYB* antisense lines, *StPHYF* RNAi lines tuberized under LD conditions and induced tuber formation, when grafted onto the stock of WT plants under LD conditions (Zhou et al., 2019).

CONSTANS and FLOWERING LOCUS T (CO-FT) pathway have been shown to regulate flowering in Arabidopsis (Amasino & Michaels., 2010; Zeveaart, 2006). CO-FT pathway, under LD conditions induces flowering. On contrary, *StCOL-1* (CONSTANS-LIKE 1) repressed tuber formation under LD (Martínez-García et al., 2002). Antisense lines of *StCOL1* tuberized under non-inductive LD conditions. *PHYB* stabilizes *StCOL1* protein to inhibit tuberization (Abelenda et al., 2016). However, day-neutral potato plants tuberizes irrespective of any photoperiod. This raises an important question that how *StCOL1* is regulated under different photoperiod? It was answered by the presence of *StCDF1* protein in potato. *StCDF1* protein forms complex with *GIGENTIA* (GI) and *FKF1*, the clock gene components and undergoes proteosomal degradation. It binds to the promoter of *StCOL1* when it's free and represses its expression to induce tuberization. Authors reported that another truncated *StCDF1* form lacking *FKF1* interacting domain does not form complex and therefore not degraded. This stabilized form of *StCDF1* represses *StCOL1*. Some day-neutral cultivar showed presence of this *StCDF* form (Kloosterman et al., 2013).

How does CONSTANS inhibit tuberization? A study by González-Schain and co-workers (2012) reported that antisense lines of *StCOL1* in andigena form tubers under non-

inductive LD. However, tuberization status under SD conditions was not affected, indicating that *StCOL1* does not play role in tuber formation under SD but, represses tuberization under LD conditions. Consistent with this study, Abelenda et al. (2016) demonstrated a key role of *StCOL1* in activation of another tuberization repressor *StSP5G* by directly binding to its promoter through TGTGGT motif. *StSP5G* negatively regulates *StSP6A* expression (Positive regulator) in leaves either by interacting with other protein partners of *StSP6A* or by inhibiting an unknown *StSP6A* activator followed by inhibition of tuber development (Abelenda et al., 2016).

1.4.1 Mobile signals in tuber development

Grafting experiments, wherein a flowering tobacco shoot as well as SD induced potato shoot scions were combined with non-induced potato stock plants resulted in tuber formation in potato stock plants (Chailakhyan et al., 1981; Gregory, 1956). This led to a possibility of transport of mobile signals from the induced scion to stock causing tuberization. Several years of research resulted in identification of mobile protein similar to FLORIGEN in Arabidopsis, and is called as tuberigen (*StSP6A*), a potato ortholog of FLOWERING LOCUS T in Arabidopsis. *SP6A* expression increases in leaves and stolons under tuber inducing SD conditions and is highly repressed under LD. Overexpression lines of *StSP6A* tuberized under LD conditions and exhibited early tuberization phenotype under SD. Silencing of *StSP6A* resulted in delay of tuber formation. As described earlier, *SP6A* was shown to be under photoperiodic control. Grafting experiments have concluded that *StSP6A* is a ‘phloem mobile signal’ protein that transports from the leaf to stolon under SD conditions (Navarro et al., 2011).

StBEL5 and *POTH1* transcription factors were shown to be SD inducible and act as positive regulators of tuberization. *StBEL5* belongs to the TALE (Three Amino acid Loop Extension) family of transcription factors whereas, *POTH1* belongs to the KNOX (Homeodomain transcription factor) family that regulate stem cell population of shoot apical meristem. Overexpression lines of *StBEL5* (Banerjee et al., 2007; Chen et al., 2003) and *POTH1* (Mahajan et al., 2012; Rosin et al., 2003) in andigena overcame inhibitory effect of LD photoperiod on tuberization and exhibited tuber earliness under SD conditions. Further grafting experiments were conducted to demonstrate the mobility of *StBEL5* (Banerjee et al., 2007) and *POTH1* (Mahajan et al., 2012) transcripts from SD induced leaves to stolons to induce tuber development. During this movement, transcript stabilization was shown to be controlled by two RNA-binding proteins, designated as *StPTB1* and *StPTB6* from

polypyrimidine tract-binding (PTB) family (Cho et al., 2015). These proteins were shown to bind to CU-rich region of 3' UTR of *StBEL5* mRNA.

Sucrose was also demonstrated as metabolic signal and transported through SUT transporters. Tuberization is highly dependent on sucrose (Gibson, 2005). Sucrose transporter-*StSUT4* silencing in andigena plants resulted in an increased sucrose transport and tuber formation under LD photoperiod (Chincinska et al., 2008). During tuber development, sucrose unloading switches from apoplastic to symplastic mode in stolons. *StSP6A* protein was found to interact with sucrose efflux transporter, *StSWEET11* and blocked leakage of sucrose to the apoplast in stolons. This action probably promoted symplastic sucrose transport. Authors concluded that mobile signals might act as mediators of source-sink partitioning in potato (Abelenda et al., 2019).

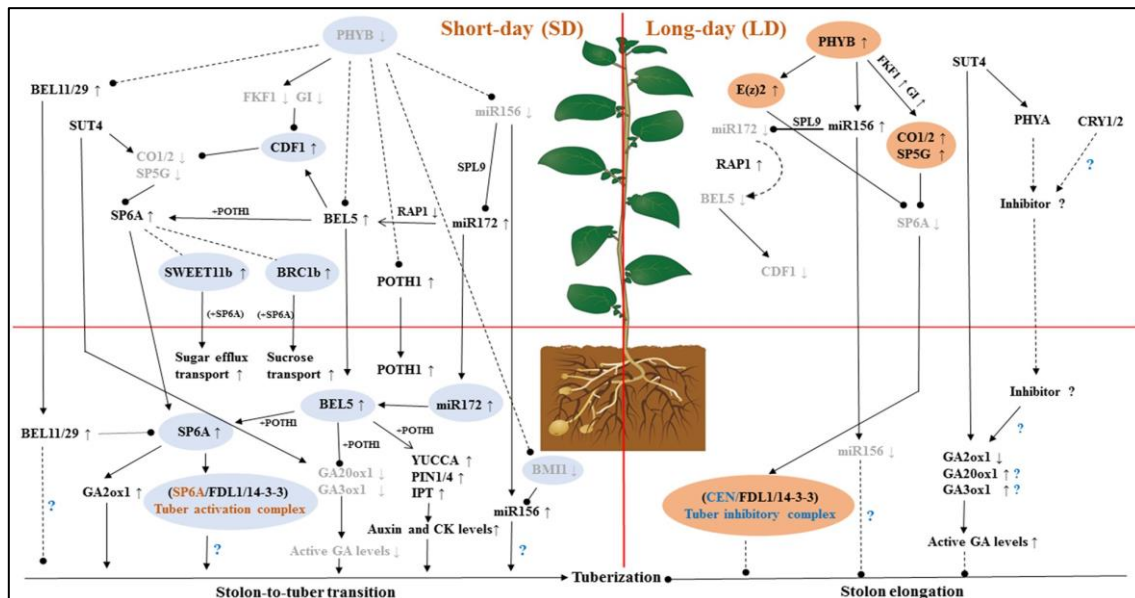


Fig. 1.4 Photoperiod dependent tuberization pathway in andigena potato plant. Short-day (SD) favors tuber development and Long-day inhibits tuberization. Lines with arrows indicate inductions and those with close end represent repression. Abbreviations are provided on page no. 6 (Adapted from Kondhare et al., 2021).

1.4.2 *StBEL5*-*StKNOX* heterodimer complex mediate tuber development

BEL1-like proteins form a tandem complex with *KNOX*-type TFs and regulate plant physiological processes. *StBEL5*-*StKNOX* complex was shown to target numerous genes involved in tuber development pathway (Lin et al., 2013). Sharma and co-workers, (2016) identified more than 10,000 *BEL5* target genes involved in growth and signal transduction, hormone metabolism, tuberization and flowering pathway from SD induced stolons of

GAS:BEL5 lines. Moreover, 92% of the differentially expressed genes showed the presence of the tandem TTGAC motifs in their upstream sequence suggesting that the BEL-KNOX heterodimer complex can bind to the tandem TGAC core motifs in their promoters region. Key tuberization genes mainly, *GA2OX1* and *GA20OX1*, *StCDF1*, *AGL8*, *LOG* family members, several *PINs* and *AUX/IAA* genes and *StBELs* including *StBEL11* and *StBEL29*, harbor TGAC motifs in their promoters acting as putative targets for the complex. *StBEL5* was also shown to activate *StSP6A* expression in SD induced leaves by binding to the TGAC motif in *StSP6A* promoter region (Sharma et al., 2016). Specifically, *StBEL5*-*POTH1* heterodimer binds to the promoter sequences of *StGA20ox1*, and represses its expression (Chen et al., 2004). Overexpression of *StBEL5*, resulted in an increased levels of *StGA2ox1* in stolons via binding to TGAC motif in its promoter sequence. As a result, bioactive GA levels decreases in stolons and stimulate tuber formation (Sharma et al., 2016). In summary, *BEL5-KNOX* heterodimer presents an important regulatory complex that governs tuberization pathway.

1.4.3 Tuber activation complex (TAC)

As suggested earlier, flowering and tuberization shares similar cues including mobile proteins signals such as florigen and tuberigen. In rice, flowering genes are regulated by Florigen Activation Complex (FAC) constituted from the interaction between Heading date 3a (*Hd3a*), 14-3-3 and a rice FD-like protein, *OsFD1* (Taoka et al., 2011). Teo et al. (2017) showed that *StSP6A* and *StFDL1*, a potato FD-like protein, interact with *St14-3-3s* to form Tuber Activation Complex (TAC). Suppression of *StFDL1* delayed tuberization, whereas *StSP6A* overexpression exhibits earliness in tuber formation in *St14-3-3* dependent manner (Teo et al., 2016). *StABL1* (*StABI5*-like 1) TF interacts with *StFT*-like proteins to form alternative TACs (aTACs) promoting flowering and tuberization (Jing et al., 2022). Authors also showed that *StABL1* regulates genes such as *AGAMOUS-like* and *GA 2-oxidase 1*. *StCEN* (*TERMINAL FLOWER 1/CENTRORADIALIS*) suppresses tuberization by competing with *StSP6A* as a part of the TAC complex (Zhang et al., 2020). Nicolas et al., 2022 reported that tuber inducing activity of *StSP6A* being regulated by *StBRC1b* gene module in axillary meristems. Jing and co-workers recently reported that *StSP3D* and *StFTL1* as graft transmissible mobile tuberization signals, which binds to the proteins of TAC complex, similar to that of *StSP6A*. This adds yet another layer of regulation to this process.

1.5 Small RNAs in plant development

Small RNAs play a central role in regulating plant growth and development. MicroRNAs and phased secondary small interfering RNAs (phasiRNAs) constitute a major category of small non-coding RNAs and perform the activities typically via post-transcriptional gene silencing (PTGS) (Fei et al., 2013).

1.5.1 Biogenesis of miRNAs and phasiRNAs

MiRNAs are of 21-24 nt small RNAs that play an important regulatory role post gene transcription. Clustering of miRNA genes from animal and plants is quite different as animal miRNAs exist in clusters throughout the genome, whereas plant miRNAs typically scattered on their chromosomes (Ha & Kim., 2014). Plant miRNA genes are transcribed by RNA polymerase II similar to a typical protein coding genes to form pri-miRNA. With the help of DCL1, pri-miRNA then cleaved to generate pre-miRNAs (60-400nt) (Fig. 1.5 A). Further, processing of pre-miRNA by DCL1 enzyme, HYL1 RNA binding protein and Serrate (SE) results in mature miRNA duplexes with a typical two bases hanging at the 3' end (Voinnet, 2009). This duplex is methylated near 3' end at 2-OH by the action of HEN1 (HUAENHANCER1) protein to prevent degradation, and it transports to the cytoplasm with the help of HASTY (plant homolog of Exportin-5). miRNAs then get loaded onto RISC (RNA inducing silencing complex) complex in cytoplasm (Zhang et al., 2022). Recent studies have shown that, entire RISC complex can be assembled inside the nucleus and then transported to cytoplasm via EXPO1. HASTY regulates pri-miRNA processing in the nucleus (Fig. 1.5 A).

Biogenesis of phasiRNA begins post cleavage of mRNAs and lncRNAs by 22-nt (but not compulsory) miRNAs (Fig. 1.5 A, B). 5' end of cleaved mRNA is degraded by a 3'→5' exonucleolytic action of the SKI2-3-8 complex. Whereas, 3' end is converted to double stranded RNA with the help of a RNA-dependent RNA polymerase (RDR6), which is recruited by either AGO1-RISC or AGO7-RISC complex (Liu et al., 2020). This assembly is aided by SUPPRESSOR OF GENE SILENCING3 (SGS3), to prevent the degradation of the 3' mRNA from a 5'→3' exoribonuclease. Resultant dsRNA is then cleaved by DCL4 protein from the 5' end of pol II derived transcript in a phased manner (varying from 21-24 nt) to yield phasiRNA duplexes (Fig. 1.5 B). Plants have at least 4 distinct classes of DCL proteins (DCL1-4). DCL1 governs the mature miRNA processing while DCL4 majorly contributes to the synthesis of endogenous siRNAs, antiviral siRNAs and phasiRNAs (Fukudome & Fukuhara., 2017). mRNA loci from which phasiRNAs are generated called as *PHAS* loci. These loci are divided

into two major groups based on their genomic location; (i) *PHAS* loci that are located in non-coding region of the genome to produce long non-coding RNAs (lncRNAs) and the ones (ii) are located within the protein coding genes. A subset of phased siRNAs from the first group (*PHAS/TAS* loci), was named as tasiRNAs based on their ability to silence transcripts from other loci (Axtell, 2013).

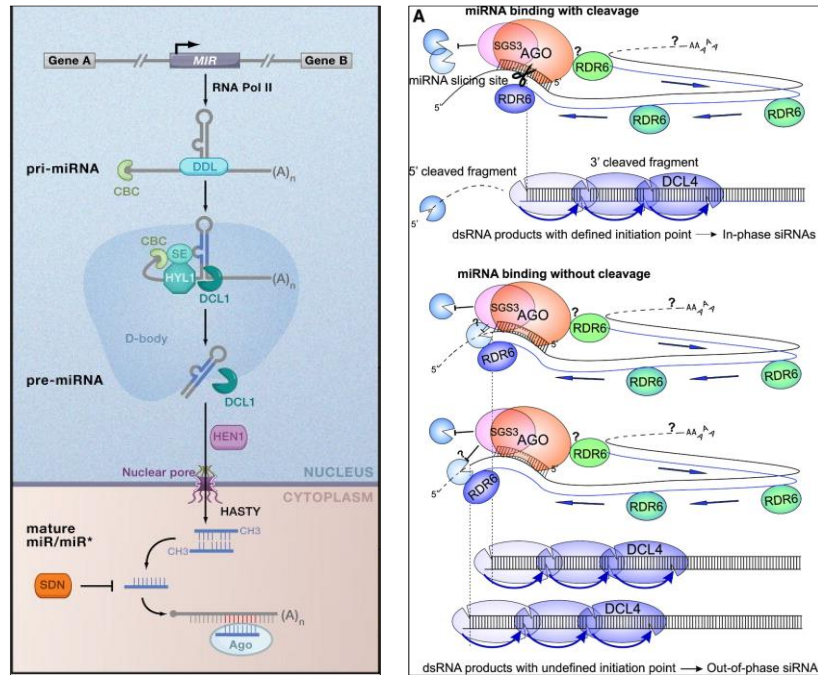


Fig. 1.5 miRNA and phasiRNA biogenesis. (A) miRNA biogenesis pathway for conserved miRNAs in plants is represented. Plant pri-miRNAs are transcribed by the action of RNA pol II and undergoes processing by DCL proteins to form mature miRNAs. (B) miRNA mediated phasiRNA (secondary siRNA) biogenesis is shown. In brief, cleaved mRNA converted to dsRNA with the help of RDR6 (in assistance with SGS3). DCL4 cleaves dsRNA in phased manner to generate phasiRNAs. (Adapted and modified from Voinnet et al., 2009 and Liu et al., 2020)

1.5.2 Mode of action

MiRNAs loaded onto the RISC complex bind to the target mRNA based on the complementary sequence and directs it to degradation. Argonaute (AGO) proteins act as an important component of this complex and contains four domains: the N-terminal domain (N), the PAZ domain, the MID domain, and the P-element-induced wimpy tested (PIWI) domain. The PAZ domain bind to the RNA and the PIWI domain has RNase H activity. The function of AGO1, 2, 4, 7 and AGO10 is shown in the gene silencing pathway of target RNAs (Zhang et al., 2015). Typically, AGO1 protein is involved in post transcriptional gene silencing (PTGS) as a main component of RISC complex with miRNAs and siRNAs. AGO7-RISC as described

earlier, participates in the formation of phasiRNAs. MiRNAs can also regulate gene expression by translational inhibition of transcript instead of mRNA cleavage (Rogers & Chen., 2013).

Similar to miRNA-RISC complexes, phasiRNAs are also loaded onto AGO1-RISC complexes and bind to target mRNAs in homology dependent manner leading to the PTGS. However, this process is not well described. PhasiRNAs exhibit homology to the region of mRNAs from which they are generated (Axtell et al., 2006). As a course of evolution, most of the phasiRNAs were primed to target gene family from which they are generated. Hence, phased siRNAs which targets mRNAs other than their gene family were named as tasiRNAs (trans-acting siRNAs). Interestingly, all the phasiRNAs generated may not be functional, as most of them are rarely detectable and do not load onto RISC complex. This is usually observed with respect to phasiRNAs and not in tasiRNAs (Liu et al., 2020).

1.6 Functions of miRNAs in plant growth and development

Plant growth and development is regulated by internal genetic information as well as external environmental ques. miRNAs act as the mediators of this regulation. Therefore, expression of miRNA throughout the plant lifecycle is necessary for proper plant growth and development (Dong et al., 2022; Reinhart et al., 2002). Numerous studies have demonstrated that miRNAs widely modulate developmental processes.

Unlike animals, plant exhibit indeterminate growth and can generate new organs throughout their life. Plant apical meristem is formed in the embryo itself and harbours the cells with an ability of self-replicate and differentiation. Shoot apical meristem (SAM) holds a potential to give rise to aerial plant organs. Shoot meristemless gene (STM), WUSCHEL (WUL) and CLAVATA (CLV) pathway plays a key role in SAM differentiation and maintenance (Schoof et al., 2000). MiRNAs play crucial role in regulating multiple genes involved in STM-WUS-CLV pathway. For example, miR394 expresses in L1 layer of SAM and diffuses to organising centre (OC) layer. It targets Leaf Curling Responsiveness (*LCR*) to inhibit its expression, subsequently results in the upregulation of *WUS* and thereby CLV3 peptide expression (Knauer et al., 2013). Even though miR394 is present in both of the cell layers, it targets *LCR* gene only in the OC layer (Fig. 1.6). Another miRNA-target pair, miR165/166-HD ZIP III transcription factors have been shown to maintain SAM development. In SAM, miR165/166 is sequestered by AGO10 and ultimately, promotes the expression of HD-ZIP III TF's to regulate SAM (Fig. 1.6). If miR165/166 does not bind to AGO10, miR165/166 is loaded onto AGO1-RISC complex and targets HD-ZIP III TF's leading to the

termination of SAM (Zhu et al., 2011). This suggests the importance of miRNAs for SAM development and maintenance.

Leaf development comprises of the formation of leaf primordium from SAM and subsequent maturing of leaf blades. Differentiation of leaf primordia requires a proper distribution of auxin (Piazza et al., 2005). MiR160 targets ARF10, ARF16 and ARF17 to regulate auxin responses during leaf development (Liu et al., 2007). A MYB transcription factor ASYMMETRIC LEAVES1/ROUGH SHEATH2/PHANTASTICA and HD ZIP III maintains ventral axis of plants. Typically, ASYMMETRIC LEAVES1 suppresses *KNOX1* expression to promote SAM differentiation to leaf (Piazza et al., 2005). *HD ZIP III* expression is maintained on adaxial side of the leaf as it is targeted by miR165/166 on the abaxial side with the help of AGO1-RISC complex (Fig. 1.6). Concentration of AGO10 is maintained on adaxial side to prevent *HD-ZIP III* degradation (Kidner & Martienssen., 2004). MiR390 and AGO7 also maintain leaf polarity through tasiRNA pathway which is described in the section (1.8.1).

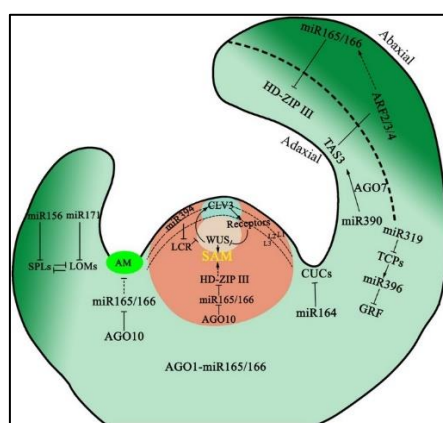


Fig. 1.6 miRNAs regulate SAM and leaf development. MiR394 regulates WUS-CLV loop in SAM formation by controlling LCR activity. MiR165/166 are sequestered by AGO10 in order to keep HD-ZIP III factors activity for SAM maintenance. Leaf primordium development is regulated via, (1) HD-ZIP III boundary formation by miR165/166 and (2) ARF2/3 regulation by TAS3 siRNAs. (Adapted from Dong et al., 2022)

Flower development in plants comprises of two processes mainly flowering induction and flower development. It also marks near end of juvenile phase and plant enters into reproductive phase. Phase transition in plants is governed by Squamosa promoter binding like transcription factors (SPL's) family (Preston & Hileman., 2013). Their expression is inhibited by miR156 and miR157 members during juvenile phase. As plant enters into reproductive

phase, expression of miRNA156 and 157 decreases as a result of which, abundance of their target proteins (SBP/SPL) increases (Xu et al., 2016). miR156 *OE* lines as well as SPL3/5 knockdown lines showed delayed flowering period, slow growth and bushy phenotypes in *A. thaliana* indicating that higher levels of miR156 keeps plant in juvenile phase (Wu & Poethig., 2006). As plant enters in the adult phase, miR156 levels decreases and allowing SPL's to perform their function. Some SPL's (*SPL9*) have been shown to promote the expression of another important miRNA, miR172. miR156-SPL-miR172 pathway has been demonstrated to control juvenile-to-adult phase transition in plants (Bhogale et al., 2014; Lian et al., 2021). miR172 regulate flower development by targeting AP2 type transcription factors (floral repressors) (Aukerman & Sakai., 2003). MiR165/166 shows tissue specific expression patterns in flowers (Fig. 1.7). However, miR165/166 constitutive *OE* lines in *Arabidopsis* lost expression specificity leading to damaged flower phenotypes (Yang et al., 2019). (Fig. 1.7).

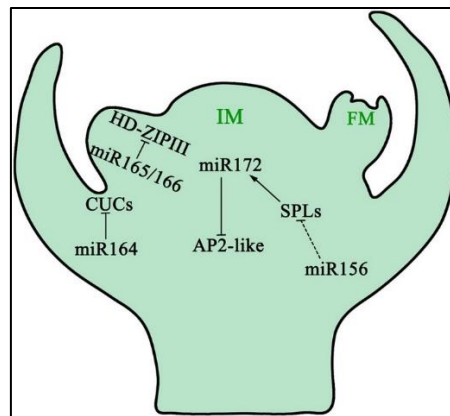


Fig. 1.7 miRNA module in flower development. MiR156 expression reduces during reproductive phase of plant resulted in the removal of inhibition on *SPL* genes. This in turn, promotes miR172 expression that targets repressor of *APETALA* gene (*AP2-like*) and triggers development of inflorescence meristem. MiR164 and miR165/166 regulate boundaries to maintain inflorescence meristem. (Adapted from Dong et al., 2022)

1.7 MiRNAs regulating tuber development

MiRNAs function as important regulators of plant growth and development, as described before. The following studies have characterized the role of few conserved miRNAs during tuber development in potato.

1.7.1 miRNA172: a positive regulator of tuberization

MiR172 was first demonstrated as a positive regulator of tuberization. Martin et al. (2009) reported that miR172 is induced under SD condition in all potato tissues with highest

expression in stem and swollen stolons. miR172 was shown to target a *RELATED TO APETALA2*-like gene, *StRAP1* which is a repressor of *StBEL5* gene. Overexpression lines of miR172 exhibit early flowering and accelerates tuber formation (Martin et al., 2009). Moreover, OE lines were able to overcome inhibitory effect of LD conditions to produce tubers even under LD condition. These findings suggest that miR172 is a connecting link acting downstream to PHYB (photoperiod), but upstream of the *StBEL5*.

1.7.2 miRNA156: an inducer of aerial tubers in potato

MiR156 maintains juvenile phase of the plant life cycle. MiR156 and miR172 crosstalk during juvenile-to-reproductive phase transitions. Both of these miRNAs regulate flower development across the plant species and found as phloem-mobile. MiR156 targets *SPL* TFs mainly *StSPL3*, -6, -9, -10, and -13, and *LIGULELESS1*. MiRNA156 levels reduces as plant grows to adult phase and expression of its target genes (*SPLs*) increases. This results in, an increase in miR172 accumulation and induction of tuber development. Therefore, miR156 overexpression lines exhibited reduced tuber yield as an effect of reduced miR172 levels. However, this lines showed profused stem branching and aerial stolons that formed tubers under SD inductions (Bhogale et al., 2014). In the same year, another study by Eviatar-Ribak et al. (2013) also reported the formation of aerial tubers in *LONELY GUY1 (LOG1)* OE lines as a result of altered cytokinin/auxin ratios in axillary meristems (Eviatar-Ribak et al., 2013). Further, miR156 was shown to be regulated by two PRC proteins, *StMSI1* and *StBMI1* in potato (Kumar et al., 2020). Whereas, *EMF1*, another PRC1 member have been demonstrated to prevent earlier flowering by repressing *SPL9* and *PRI-MIR172* genes (Picó et al., 2015). These results suggest that miR156-miR172 crosstalk is crucial for tuberization and both of these miRNAs are under tight control by epigenetic modifiers.

1.7.3 miRNA390 in tuber development

Calcium dependent protein kinases (CDPKs) are important regulators of plant signaling which relay calcium signals into physiological processes by phosphorylating substrates involved in hormone signaling as well as stress responses pathway. Potato *StCDPK1* has been demonstrated to phosphorylate hydrophilic loop of potato PIN-FORMED (*StPIN4*) auxin efflux carrier *in vitro* (Santin et al., 2017). PIN proteins determine the direction of auxin flow and are controlled by reversible phosphorylation of PIN hydrophilic loop (PINHL). Hence, it was suggested that *StCDPK1* could regulate auxin transport in stolons via regulation of *StPIN4*. miR390 has been demonstrated to target *StCDPK1* by transcriptional as well translational

inhibition. miR390 and *StCDPK1* exhibited inverse expression pattern in potato leaves and tubers (Santin et al., 2017). This study suggested that small RNAs targeting kinases provide additional control on phosphorylation dependent developmental processes in plants.

1.7.4 SES-SP6A regulation in tuber formation

FLOWERING LOCUS T (*SP6A*) in potato acts as a positive regulator of tuberization. Lehretz et al., 2019 demonstrated small RNA-mediated regulation of *SP6A* during tuber development. Overexpression lines of potato expressing codon-optimized version of *SP6A*^{cop} exhibited extremely early tuberization phenotype as compared to the plants expressing an unmodified *SP6A* version. This observation leads to the identification of putative small RNA targeting *SP6A* gene, named SES (Suppressing Expression of *SP6A*). SES expression increases under heat stress, whereas *SP6A* reduces under heat. SES knockdown lines exhibited increased *SP6A* levels and tuberizes even under continuous heat conditions compared to WT, which does not tuberize. Thus, the SES-*SP6A* module enables additional regulation of the tuber development pathway and provides a promising strategy for breeding heat-tolerant potatoes (Lehretz et al., 2019).

1.8 PhasiRNA pathways in plant development and defense responses

Plant phasiRNAs have been shown or predicted to function in various plant biological processes (Fei et al., 2013; Liu et al., 2020). Next generation sequencing approaches have resulted in identifying a plethora of phasiRNAs and *PHAS* loci from different plant species, yet their function is unknown. Very few phasiRNA pathways are well characterized in plants.

1.8.1 Conserved miR390-TAS3-ARF3 module regulate plant development

Amongst the plant *TAS* genes, *TAS3* is well studied. *TAS3* transcript exhibits two miR390 sites and generates tasiRNAs via “two-hit” mechanism (Axtell et al., 2006). One of the tasiRNAs, tasiR-ARFs target auxin responsive factors (ARFs). Auxins play important role in plant growth and development. The ARF family of TF’s regulates gene expression either by activating or repressing auxin-regulated genes via protein interactions with AUX/IAA family members. Thus, miR390-*TAS3*-*ARFs* pathway is well demonstrated in regulation of leaf morphogenesis, developmental patterning, somatic embryogenesis and lateral root development (Adenot et al., 2006; Fahlgren et al., 2006; Lin et al., 2015; Marin et al., 2010). Diverse phenotypic variations have been observed if any of the pathway component is disordered. TasiR-ARFs expresses in the two most adaxial cell layers along with the expression

of AGO7. It moves to the cell layers on abaxial side forming gradient (Fig. 1.8) (Chitwood et al., 2009). As tasiRNAs targets *ARF3* transcripts, rendering *ARF3* complementary gradient from abaxial to adaxial side. In maize, the mutation of *LEAFBLADELESS1 (LBL1)*, which encodes an ortholog of *SGS3* resulted in abaxialised leaves due to inability of plant to produce tasiRNAs (Fig. 1.8) (Singh et al., 2018). Loss of function of AGO7 or DCL4 causes a wiry leaf syndrome in tomato (Yifhar et al., 2012) whereas *ragged seedling2 (AGO7)* mutant in maize, displays cylindrical leaves with dorsiventral polarity, as plant could not generate tasiR-ARFs. These results support the fact that tasiR-ARFs produced in adaxial layer of the leaf is necessary to determine the leaf polarity.

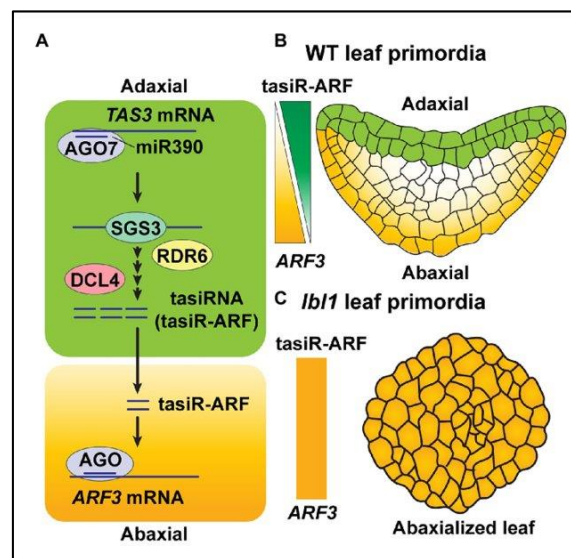


Fig. 1.8 TasiRNA mediated leaf polarity establishment. (A) MiR390 expresses throughout in the leaf. AGO7 expresses on adaxial side leads to the generation of 21-nt tasiRNA, tasiR-ARF. It moved to abaxial side to target *ARF3* and represses its expression. (B) WT leaf primordia showing gradient of TasiR-ARF synthesized on adaxial side targets *ARF3* mRNA forming the concentration gradient of *ARF3* in opposite direction. (C) *lbl1* mutant in maize was defective in tasiRNA production. Defective tasiRNA and *ARF3* gradient resulted in abaxialised leaf phenotype. (Adapted from Yan et al., 2022)

1.8.2 miR828-TAS4-MYB module in anthocyanin biosynthesis

Another example of evolutionary conserved phasiRNA pathway is miR828-TAS4-MYB regulating anthocyanin biosynthesis across dicots and monocots. miR828 in Arabidopsis triggers the cleavage of *TAS4* transcripts to produce siRD81(-) that targets MYB transcription factors (Xia et al., 2012). MYB gene family is one of the largest regulators of gene expression in plants. These are mainly involved in regulating primary and secondary metabolic reactions, and responses to environmental stresses by regulating entire gene network (Liu et al., 2015).

MYB TFs are itself under transcriptional and post-transcriptional control. The latter is controlled by the action of miRNAs and siRNAs. miR828, miR159 and miR858 have been shown to target at least 13 MYB genes. miR828 targets MYB genes and generate secondary siRNAs to reinforce the regulation of MYB gene family (Fig. 1.9). In grapes, this pathway was demonstrated to target *VvMYBA6* and *VvMYBA7* that have been shown to regulate enzymes from flavonol and anthocyanin biosynthesis pathways (Tirumalai et al., 2019). Some of the MYB TFs also regulate lignin biosynthesis and fruit development pathways. *GhMYB2* targeting in cotton (*Gossypium hirsutum*) by miR828 play an important role in fiber development (Guan et al., 2014). miR828-TAS4- siRD4(-)-MYB12 pathway was shown to develop purple tuber skin and flesh colour in potato (Bonar et al., 2018). This pathway was active in tubers causing the low expressions of *StMYB12* and *R2R3-MYB* factors resulted in increased anthocyanin production.

1.8.3 miR9678-WSGAR module in seed germination

Regulation of seed germination is important for plants to adapt various environmental conditions. During dormancy, seed germination is inhibited under unsuitable environmental conditions, which enhances their shelf life. However, few plant seeds tend to germinate early, following environmental conditions such as rain and cold. This condition is termed as pre-harvest sprouting (PHS) causing substantial losses of grain yield and quality. Seed germination is a result of increased gibberellin signalling. Wheat specific miRNA, miR9678 has been proven its suitability for improving PHS resistance. MiR9678 targets a long non-coding RNA, WSGAR and triggers phasiRNA generation from lncRNA to delay seed germination (Fig. 1.9). Targets of these siRNAs have not been determined yet. Moreover, miR9678 OE lines of wheat showed downregulation in GA biosynthesis gene, and reduces GA signalling by upregulation of GA catabolism genes. Further, proteins associated with ABA signalling binds to miR9678 promoter to enhance its expression thus suggesting that miR9678 dependent generation of phasiRNAs regulate seed germination by modulating GA/ABA signalling. As the target genes of phasiRNAs have not been identified, the molecular mechanism for phasiRNAs function is still unclear (Guo et al., 2018).

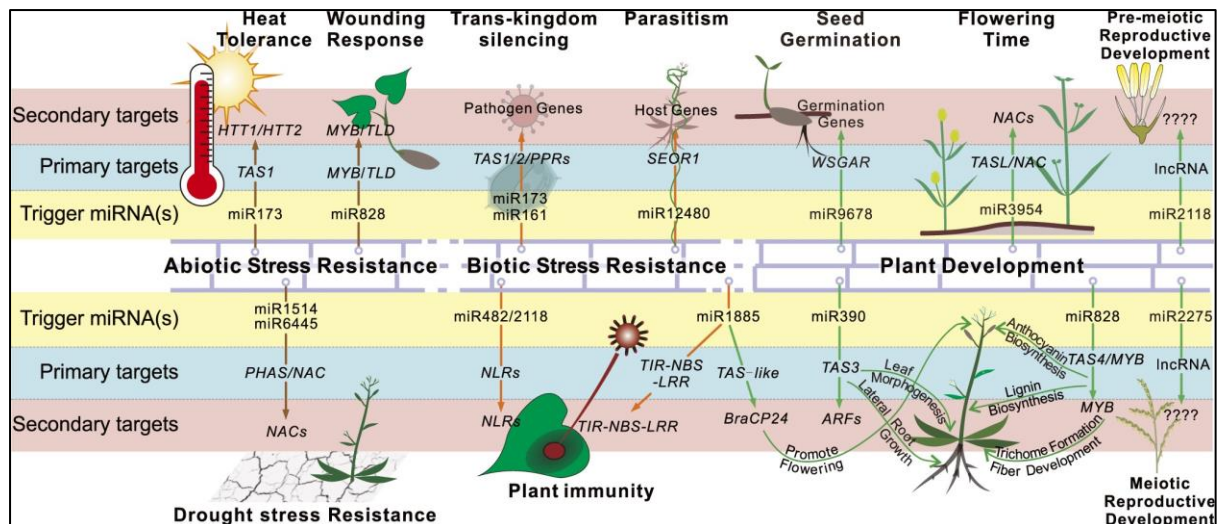


Fig. 1.9 PhasiRNAs in plant development and stress responses. Gene regulatory pathways led by phasiRNAs are represented. Regulation is conducted on three levels. First level (yellow) by a trigger miRNA, second level (blue) primary miRNA target i. e. *PHAS/TAS* loci and third level is phasiRNA targets regulating processes (Adapted from Liu et al., 2020).

1.9 PhasiRNAs in *Solanaceae* family

Solanaceae family harbours a unique small RNA populations associated to the diversity of members. Recent study by Baldrich and others, 2022; presented a comprehensive phasiRNA analysis from available sRNA sequencing datasets of *Solanaceae* family members. All the members of phasiRNA biosynthesis pathway such as AGO's, DCL's pathway is found to be conserved in *solanaceae* plants. Most of the *PHAS* loci are located in genes encoding disease resistance-related NB-LRRs, pentatricopeptide repeat (PPR) proteins, MYB TF's, and *TAS* loci. Interestingly, potato, tomato and capsicum exhibits two to three times more *PHAS* loci than that of *Nicotiana* and petunia. Majority of them are derived from disease resistance-related genes that are predicted to be triggered by the miR482/miR2118 superfamily (Baldrich et al., 2022).

1.9.1 miR482/miR2118 family regulates *NB-LRR* genes in *Solanaceae*

As described earlier, miR482/miR2118 family triggers phasiRNAs comprising the majority of small RNA population in potato and tomato (Zhang et al., 2022). Members of miR482 family target NBS-LRR disease resistance gene transcripts leading to the production of secondary siRNAs in RDR6 dependent manner. PhasiRNAs generated from these genes further target other mRNAs of defense related genes. This miR482 mediated signaling cascade is suppressed in plants infected with pathogens to increase the expression of defense related

genes. NBS-LRR proteins are often associated with effector-triggered immunity against pathogens. This increased NBS-LRR proteins post infection, can also help plant to accelerate defense signals against secondary infections. Expression of defense genes coupled to the RNA silencing system adds another layer to the defense and counter-defense interactions between plant and pathogen. This pathway may also enables plant to reduce the cost associated with the maintenance of individual NBS-LRR genes. Instead, the low level expression of these genes is maintained by miR482/miR2118 family members allowing diversification of the NBS-LRR gene family without posing a cost to the plant (Shivaprasad et al., 2012).

1.9.2 PhasiRNA-gibberellin module in potato during PVY infection

Potato virus Y (PVY) belongs to the potyviridae family and is the most harmful pathogen for potato crop, causing a worldwide huge loss to tuber quality and quantity. Cv. Désirée, which is tolerant to PVY^{NTN}, is most widely grown potato variety. PVY virus can infect this plant and spread systematically, but there are no visible or mild symptoms. With the help of this variety, Križnik et al. (2017) identified the role of small RNAs in regulating this complex immune signaling network that controls the tolerant response towards PVY infection. Notably, the expression of two small RNAs, miR167 and phasiRNA931 increases post infection at early time points. Both of them are predicted to cleave gibberellin biosynthesis genes *GA20-oxidase* and *GA3-oxidase*, respectively. Here, lower GA signaling is found to be correlated with less disease severity. This network is attenuated in salicylic acid depleting conditions, indicating that the phasiRNA-gibberellin defense network is salicylic acid dependent (Križnik et al., 2017). This intertwining of two different regulatory networks show balance between developmental processes, defense signaling and plant stress responses.

1.10 Identification of small RNAs from potato: previous studies.

Various small RNAs and their targets from model plants have been reported with the help of bio-informatic and experimental approaches. Zhang and co-workers (2009) predicted 48 miRNAs, whereas Yang and co-workers (2009) reported 71 miRNAs using potato ESTs (Expressed Sequence Tags), GSS (Genome Survey Sequences). Using comparative genome strategy, Xie et al (2011) identified 202 miRNAs belonging to 78 miRNA families from potato. They also predicted targets for few miRNAs and validated them by real time PCRs. Potato genome information was made available in 2011 (“Genome Sequence and Analysis of the Tuber Crop Potato,” 2011). With the help of available genome Zhang and co-workers (2013) characterized miRNA populations from leaves and stolons of photoperiod dependent variety

of potato *Andigena*. This study represented 28 conserved miRNA families and 120 potato-specific miRNA families (Zhang et al., 2013). Lakhotia et al (2014) reported 89 conserved miRNAs and 147 potato-specific miRNAs followed by their target prediction. However, the study was limited to photoperiod non-responsive potato cultivar- Chandramukhi. Altogether, these studies have shown the existence of complex miRNA repertoire in potatoes and suggested their possible role in the tuber development pathway; nevertheless, these studies were only restricted to miRNAs.

1.11 *Solanum tuberosum* ssp. *andigena* as a model system

To investigate the function of small non-coding RNAs in stolon-to-tuber transitions, *Solanum tuberosum* ssp *andigena* was used as a model system. It is a photoperiod dependent diploid potato variety. Andigena plants tuberize under the strict short days (SD) and does not form tubers under long days (LD) photoperiod (Fig. 1.10). Therefore, it serves as a good model system to study photoperiod dependent tuberization. We have also used potato cultivar Desiree in the later part of the study to test photoperiod dependency of miRNA function. Desiree is a day-neutral (photoperiod insensitive) cultivar that forms tubers under LD conditions.

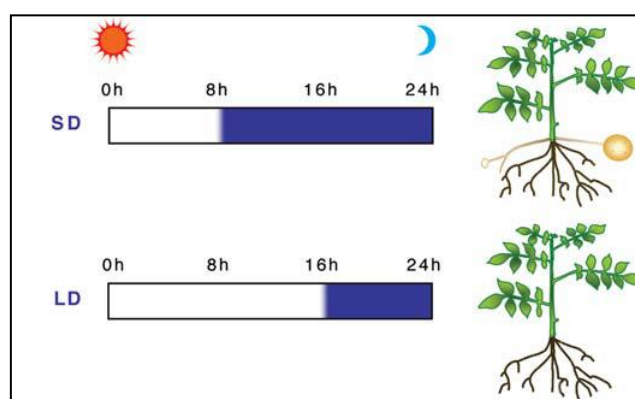


Fig. 1.10 *Solanum tuberosum* ssp. *andigena*: a model potato plant. Schematic representation of andigena plant tuberizing under short-day (8h light, 16h dark) conditions and not in the Long-day (16h light, 8h dark) (Adapted from Jackson et al., 2009).

1.12 Hypothesis and Objectives

Stolon-to-tuber transition is a crucial part of tuberization process (tuber development). From the literature review on tuberization pathway, we observed that numerous studies have well described the key regulators (photoperiod, transcription factors, mobile RNAs, proteins and hormones) governing this process but only few of these reports, have investigated the mechanism of stolon-to-tuber transition. Our understanding in this regard is still limited.

Phytohormones such as gibberellin (GA) and auxins were demonstrated to drive stolon transitions. Particularly, reduced GA levels and increased auxin concentration at the onset of tuberization, is a pre-requisite switch for the stolon converting into a tuber. This GA:auxin ratio causes a change in the cell division pattern in stolons from longitudinal to radial to help in cell expansions. The mechanistic basis for this regulation is still unclear. Also, during this process, the metabolic flux in stolons switches from cell growth phase to starch storage phase, wherein the mode of sucrose unloading is shifted from apoplastic to symplastic mode, mediated by blocking of sucrose transporters that is necessary for starch synthesis. How does plant synchronize the on-loading of sucrose in phloem cells and its off-loading into stolon cells that aligns with the cellular differentiation program would be interesting to investigate. Further, various enzymes have been shown to control complex metabolic network in stolons suggesting that tuber development is indeed a multifaceted phenomenon. It is fascinating to understand how does plant co-ordinate these dynamic changes occurring in stolon during tuber formation. From our literature survey, we perceived that small RNAs function as a key drivers of various plant developmental processes by governing the gene regulatory networks. Thus, we speculate that the mechanism of stolon-to-tuber development could be controlled by transcription factors as well as small RNAs, in a multi-level gene regulatory modules. To gain further insights, we asked the following questions, (i) How does plant maintain the GA and auxin hormonal balance throughout this transitions process to control cell divisions? And (ii) Does potato plant employ regulatory pathways to fine-tune the developmental transition process? An in-depth approach is necessary to understand the regulation of tuber transitions and it's fine-tuning via inter-locking regulatory loops. Previous studies on small RNA sequencing in potato, identified plethora of miRNAs with potential role in tuberization. However, majority of them lack the effect of photoperiod on tuber formation as these studies were carried out in a day-neutral cultivar. As day-length is a critical factor influencing tuber development, we hypothesize that profiling of sRNAs from early stolon-to-tuber transition stages under differential photoperiodic (SD/LD) conditions could unravel the key small RNAs that are specific for tuberization and might regulate hormonal networks operating during the tuber development process. Further, we have identified interesting candidate small RNAs that target GA and auxin hormone related genes, making us to believe the possibility of inter-locked gene regulatory mechanism governed by novel small RNAs.

Hence, we determined the following objectives for this study.

1. To identify small RNAs from early tuber transition stages of potato under differential photoperiod conditions.
2. To decipher the role of novel phasiRNA [siRD29(-)] in stolon-to-tuber transition stages.
3. Investigating the function of a novel potato-specific miRNA (miRNA72) during potato development.

Chapter 2

Identification of small RNAs and target genes from stolon-to-tuber transition stages of potato under photoperiodic conditions

2.1 Introduction

Small non-coding RNAs play a central role in regulating various facets of plant growth and development. MicroRNAs and phased secondary small interfering RNAs (phasiRNAs) constitute a major category of small RNAs, and perform their activities via post-transcriptional gene silencing (PTGS). miRNAs are synthesized from their precursors, whereas, *TAS/PHAS* loci encode transcripts, which are cleaved by specific miRNAs to generate phasiRNAs (Voinnet, 2009; Fei et al., 2013). MiRNAs regulate nearly all developmental processes, such as shoot apical meristem development and maintenance, leaf morphogenesis, juvenile-to-adult phase transition, flowering, and root development (Dong et al., 2022). A subset of phasiRNA, known as trans-acting siRNAs (tasiRNAs), are known to function in plant growth and stress responses (Liu et al., 2020). Major *TAS* loci members are shown to be conserved across plant species. For example, the miRNA390-*TAS3-ARF* pathway was shown to be well conserved across the plant lineage, and plays an important role in plant development, including developmental timing and patterning, leaf morphogenesis, lateral root growth, and somatic embryogenesis (Xia et al., 2017). PhasiRNAs were also demonstrated to regulate hormone metabolism genes. For example, a wheat-specific miRNA, miR9678, targets lncRNA (WSGAR) to produce phasiRNAs that regulate the germination process in wheat by controlling the expression of GA metabolism genes (Guo et al., 2018). Given the large number of *TAS/PHAS* loci and population of phasiRNAs, their precise functions in plants are not well understood (Liu et al., 2020).

Tuber development in potato is an excellent example of a multifaceted phenomenon where environmental, biochemical and genetic factors work in a concerted manner (Hannapel et al., 2017; Kondhare et al., 2020, 2021). In this process, under tuber-inducing conditions, a belowground modified stem (stolon) transverse through developmental transitions from swelling at the sub-apical region, the development of the mini-tuber, and tuber maturation. At the onset of tuber formation, a dynamic change in the hormonal regulatory network of gibberellin and auxin controls the plane of cell division from longitudinal to transverse, which results in swelling in sub-apical regions of the stolons (Xu et al., 1998). Further, starch and protein accumulate in the cells to favor tuber maturation. During this transition process, the metabolic pathways within stolons shift from promoting cellular growth to facilitating the storage of starch, which further establishes an intricate metabolic network of enzymes (Tiessen et al., 2002; Van et al., 2017). Understanding how plants coordinate these dynamic changes occurring in stolons during tuber formation is intriguing. We speculate that novel gene

regulatory pathways could govern these critical changes during stolon-to-tuber development. An in-depth approach is necessary to better understand the fine-tuning of this stolon-to-tuber transition process. As small RNAs have been demonstrated as critical drivers of this developmental process (Fei et al., 2013; Liu et al., 2020; Dong et al., 2022), we believe that small non-coding RNAs could act as major players driving the gene regulatory networks of stolon-to-tuber transitions.

Two earlier studies have reported sRNA profiling from potato stolons (Lakhotia et al., 2014; Zhang et al., 2013). Though a thorough analysis was carried out to identify miRNAs involved in the tuber formation, the effect of photoperiod on the early stages of stolon development was not considered (Table 2.1). Photoperiod is the widely studied external factor having the most critical influence on tuberization. Usually, short days (SD) favour tuber formation, whereas, long days (LD) have repressive activity (Rodríguez-Falcón et al., 2006). Photoperiod-dependent CONSTANS-FLOWERING LOCUS T (CO-FT) pathway have been demonstrated to regulate flowering in Arabidopsis (Corbesier et al., 2007) and tuberization pathway in the potato (Martínez-García et al., 2002). To identify the potential involvement of sRNAs in the early stages (4, 7, and 10 days) of stolon-to-tuber transitions in a photoperiod-dependent manner, we undertook a deep-sequencing approach using stolon samples of a photoperiod-sensitive potato cultivar (*S. tuberosum* ssp. *andigena*) under long-day (LD) and short-day (SD) photoperiodic conditions.

Table 2.1 Summary table for small RNA sequencing studies from potato.

References	Key findings
Zhang et al. (2009)	48 miRNAs were predicted through expressed sequence tags (ESTs) and genomic survey sequences (GSS).
Yang et al. (2009)	71 miRNAs were predicted from potato.
Xie et al. (2011)	202 potential miRNAs were identified using comparative genome strategy.
Zhang et al. (2013)	28 conserved and 120 potato-specific miRNA families were identified by high-throughput sequencing of leaf and stolons.
Lakhotia et al. (2014)	89 conserved and 147 potato specific miRNAs were identified from tuber developmental stages in a day-neutral cultivar.

2.2 Material and methods

2.2.1 Plant growth conditions and stolon sample harvest

In this study, the photoperiod-responsive potato cultivar - *Solanum tuberosum* ssp. andigena 7540 that tuberizes under short-day conditions (SD; 8 h light / 16 h dark) was used. Plants were grown in soil for eight weeks under long-day conditions. (LD; 16 h light / 8 h dark) Further, half of the plants were transferred to the tuber-inducing SD conditions, whereas the remaining plants were maintained under LD photoperiod in a growth chamber (Percival Scientific). Stolon apices were harvested at the end of 4th, 7th and 10th days of SD/LD induction as these samples represented early stolon stages of tuber development. Stolon samples from four independent plants out of eight plants were pooled to form two biological replicates at each time point.

2.2.2 Sample preparation for small RNA sequencing

A total of six samples from SD/LD induction were processed in duplicates. Altogether, 12 RNA libraries were sequenced. Total RNA was isolated from crushed tissues using RNAiso Plus (DSS TAKARA) as per the manufacturer's instructions. RNA concentration was estimated with NanoDrop spectrophotometer (Thermo scientific-M1000). RNA integrity was checked on the Agilent Bioanalyzer chip. Small RNAs were purified with mirPremier™ microRNA Isolation Kit, and sRNA profiles were also checked on the Bioanalyzer. Further, twelve sRNA libraries were prepared independently, sequenced on the Illumina platform, and the FastQC reports were obtained.

2.2.3 Processing of raw reads and miRNA predictions

Raw reads were processed with miRPro, an open-source standalone program (<https://sourceforge.net/projects/mirpro/>). The quality of raw reads was examined by FASTX-Toolkit and filtered for high quality, keeping a quality score ≥ 20 . The rRNA and tRNA reads were filtered, and adapter sequences were trimmed off. Finally, clean reads were collapsed with expression numbers and counted. Collapsed reads were then mapped to the known pre-miRNAs (hairpin sequences from miRBase) using the Novoalign tool with no mismatches, and miRNA quantification was carried out following the protocol described in (Shi et al., 2015). For novel miRNA predictions, miRPro uses a similar algorithm as in miRDeep2 (Friedländer et al., 2012) with minor modifications. We also used ShortStack (Axtell, 2013) program for miRNA prediction to verify the results obtained.

2.2.4 Differential expression analysis for miRNAs

R package, DESeq2 tool (Love et al., 2014) was used to identify differentially expressed (DE) conserved and novel miRNAs between LD and SD photoperiod at three early stages of stolon development (LD4 vs SD4, LD7 vs SD7, and LD10 vs SD10). We had a fourth category to distinguish between early and late stolon stages i.e., SD4 vs SD10. Read count tables provided by miRPro were given as input for DE analysis. MA scatter plots were prepared for DE miRNAs at three LD vs SD pairwise comparisons (namely, LD4 vs SD4, LD7 vs SD7, and LD10 vs SD10) as well as SD4 vs SD10.

2.2.5 Identification of putative miRNA targets and Gene Ontology

FASTA files with miRNA sequences were given as input for target predictions with psRNATarget tool (plantgrn.noble.org/psRNATarget) (Dai et al., 2018) using default parameters and expectancy-value ≤ 3.0 . Further, Gene Ontology (GO) annotation was performed using the Blast2GO software v1.3.3 (Conesa et al., 2005). Nucleotide sequences of miRNA targets were cloud-blasted against a non-redundant (nr) protein database (NCBI) using the BlastX program in the Blast2GO software (parameters for cloud-blast: sequence length ≥ 100 bp; number of blast hits, 20; HSP length cut-off, 33; and e-value, 10). GO term mapping was done with a sequence length ≥ 100 bp. Further, annotations were performed using the Annotation tool by keeping the following parameters: sequence length ≥ 150 bp; e-value Hit Filter set to 3; annotation cutoff set to 25; GO weight set to 5.

2.2.6 Cleavage site mapping

A modified 5' RNA Ligase-Mediated Rapid Amplification of Complementary DNA Ends (5' RLM-RACE) was performed using the First Choice RLM-RACE kit (Ambion) to map the cleavage site of miRNAs on predicted targets. Briefly, total RNA from stolon samples at 4, 7, and 10 d LD/SD time points were mixed in equal concentration, and ligated to the 5' RNA adapter. cDNA synthesis was performed with SSIV RT using oligo (dT) primers. Two sets of PCRs were performed with adapter-specific forward primers and gene-specific reverse primers (Table 2.3). PCR amplicons from secondary nested PCRs were then cloned in a sub-cloning vector pGEM-T-easy, and sequence verified to identify miRNA cleavage sites on target gene transcripts. Target of miR160, i. e. *ARF10* in Potato was used as a positive control in the 5' RLM-RACE assay.

2.2.7 cDNA synthesis and quantitative PCRs

To validate miRNAs and their targets, total RNA was isolated using TRIzol reagent from the powdered aliquot of the stolon samples. miRNA-specific complementary DNA (cDNA) synthesis was carried out using 4 µg of total RNA in a 40 µl reaction, Superscript IV Reverse Transcriptase (SSIV-RT Invitrogen) and miRNA-specific stem-loop primers using previously published protocol (Varkonyi-Gasic et al., 2007). Quantitative PCR reactions (qRT-PCR) were performed for 14 differentially expressed miRNAs (conserved and novel) using miRNA-specific forward primer (FP) and universal reverse primer (RP) (Table 2.3) on a CFX96 Real-Time System (Bio-Rad). Small nuclear RNA *StU6* was used as reference gene for normalization. All the PCR mixtures were prepared with TAKARA SYBR[®] green master mix (Takara) and incubated at 95°C for 30s, followed by 40 cycles of 95°C for 5s, 56°C for 15s and 72°C for 15s. PCR specificity was checked by melting curve analysis, and data was analysed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Further, PCR products were run on 3% agarose gel to confirm the presence of a band of expected size. Similarly, for target validation, 4 µg of total RNA was reverse-transcribed in a 40 µl reaction with oligo (dT) primers and qRT-PCR was carried out using respective gene-specific FP and RP (Table 2.3). *EIF3e* was used for reference gene for normalization of target genes.

2.2.8 PhasiRNA predictions and their putative targets

To identify Phased siRNA-producing loci (*TAS-like* loci), sRNA sequences from all 12 stolon libraries were mapped to potato reference genome (*Solanum tuberosum* v4.03) using TA-SI prediction tool from UEA small RNA workbench (version 3.2; <http://srna-workbench.cmp.uea.ac.uk/>) with default parameters at $p < 0.0001$ (Stocks et al., 2018). This tool utilizes an algorithm as described in (Chen et al., 2007). Further, to identify miRNA triggers of the *TAS-like* loci, these sequences were given as an input to psRNATarget tool (plantgrn.noble.org/psRNATarget), and potential phasiRNAs producing loci were predicted. The genomic location of loci were retrieved from the PGSC database, to categorize them as exonic and intergenic loci. PhasiRNAs from these loci were subjected to psRNATarget tool ($E < 3.0$) to identify putative target genes. GO analysis was performed to annotate their function as described before.

Sequences comprising 300bp upstream and 300bp downstream of each *TAS-like* locus region (251bp) were retrieved from PGSC database (Xia et al., 2013, 2017) and a total of 851bp sequence of each *TAS* locus was given as an input to the psRNATarget finder to identify *loci*

triggered by miRNAs. Subsequently, phasiRNAs generated from miRNA-triggered loci with abundance value ≥ 10 , were subjected to psRNA Target finder ($E < 3.0$). Putative target genes related to tuberization were identified.

2.2.9 Validations of *TAS-like* loci

Two putative *TAS-like* loci present within the exonic (genic) regions were validated. Quantitative PCR analysis using primers on either side of the predicted cleavage sites in their transcript sequences was carried out from stolon samples (Table 2.3). *EIF3e* was used for normalization. Similarly, stem-loop RT-PCR was performed for the detection of phasiRNAs, generated from these loci (two from each locus) as described in 2.2.6 (Varkonyi-Gasic et al., 2007). Amplified PCR products were then cloned into a sub-cloning vector pGEM-T Easy (Promega) and sequence confirmed.

To identify the presence of conserved *TAS* loci in our data, we aligned *TAS3* sequences from *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Solanum lycopersicum* to all *TAS* loci predicted in our analysis using BLAST. Global alignment of *TAS3* loci was carried out using MUSCLE (Edgar, 2004). Conserved sequences were highlighted using the BoxShade server, version 3.21, written by Hofmann and Baron (Albà, 2000).

2.2.10 Accession numbers

Table 2.2 The accession numbers for the transcripts analyzed in this chapter:

Sr. No.	Gene Name	Accession	Source
1	<i>StARF10</i>	PGSC0003DMT400020874	PGSC
2	<i>StGRAS</i>	PGSC0003DMT400031475	PGSC
3	<i>StGAMYB</i>	PGSC0003DMT400058426	PGSC
4	<i>StTm2</i>	PGSC0003DMT400051269	PGSC
5	<i>StPHO2</i>	PGSC0003DMT400076435	PGSC
6	<i>StARF2</i>	PGSC0003DMT400036765	PGSC
7	<i>StARF3</i>	PGSC0003DMT400081282	PGSC
8	<i>AtTAS3b</i>	AT3G17185.1	TAIR
9	<i>StReplication factorC</i>	PGSC0003DMT400004500	PGSC
10	<i>StTCP2</i>	PGSC0003DMT400051252	PGSC
11	<i>St-metal dependent phosphorylase HD domain containing protein</i>	PGSC0003DMT400079594	PGSC

12	<i>StExotosin family containing protein</i>	PGSC0003DMT400005712	PGSC
*PGSC- Potato Genome Sequencing Consortium (http://spudb.uga.edu/)			
* TAIR – The Arabidopsis Information Resource: (https://www.arabidopsis.org/)			

2.2.11 Primer Sequences

Primers used in this chapter are listed in Table 2.3

Table 2.3. List of primers used in this study.

Sr. No.	Primer name	Sequence (5'→3')
1	miR477b STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACCAGAAG
2	miR477a STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACGAGAAG
3	miR319 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACGAAGGG
4	miR399g STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACTAGGGC
5	miR482d STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACGGCATG
6	miR479 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACGAGTGA
7	n-miR147 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACAGGCTA
8	n-miR206 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACTCGGCA
9	n-miR93 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACGGAATC
10	n-miR40 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACAGGCTT
11	n-miR139 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACCATTTC
12	n-miR302 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACTTTCCC

13	U6 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACTTGGAC
14	miR477b FP	CAGCGGTACTCTCCCTCAAAGG
15	miR477a FP	TAGAGGTCTCTCCCTCAAGGG
16	miR319 FP	CAGCGGTTTGGACTGAAGGGTT
17	miR399g FP	TACAGTCGCCAAAGGGGAGCT
18	miR482d FP	TACAGTTCTTGCCTACACCGCC
19	miR479 FP	CAGCGGTTGAGCCGAACCAATA
20	n-miR147 FP	GCGGCGGTATCATCTCGAATTTTGAA
21	n-miR206 FP	TATCGGTCAGCCAGGGATGACT
22	n-miR93 FP	CAGCGGTAGCTGCTGACCTATG
23	n-miR40 FP	CAGCGGTAGGGTCTGTAGATGACTT
24	n-miR139 FP	CGGCGGTACTGGAGGGCATAAATAT
25	n-miR302 FP	ACGGCGGTGTTCAAGAAAGTTGT
26	U6 FP	GACACGCACAAATCGAGAAATG
27	Universal RP	AGTGCAGGGTCCGAGGT
28	StARF10 FP	GTCCAGCAGTCCTTTCTGTTGTTT
29	StARF10 RPi	GCTGCAACACGCTGGAAACTT
30	StARF10 RPo	GGCTGACCGAAGAGTAAGAACC
31	StGRAS1475 FP	CGGCGTGAATAAAAAGCCGTGAG
32	StGRAS1475 RPi	CGTGTAACGTACCGGAAGCAGC
33	StGRAS1475 RPo	GCTAGAACATCTCCCTGATGGTGT
34	StGAMYB8426 FP	GCAACTCCTCTTCAGAGCCCA
35	StGAMYB8426 RPo	GATGTCTCGTTCCCTTGGTGTGA
36	StGAMYB8426 RPi	GTTGCTAGGTGACAGACTACCGGAT
37	StTm2 1269 TAS FP	TGCCCCGTCTGGGGAAAA
38	StTm2 1269 TAS RPi	GTCCAACCTTCCTTGGCTATGCC
39	StTm2 1269 TAS RPo	AACATCCCAAATGTCATCTAGGAC
40	StTm2 siR1 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACACACTC
41	StTm2 siR3 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACCTTGGC
42	StPHO2 -siR3 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACGCCGTG

43	StPHO2 -siR4 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACGCACTT
44	StTm2 siR1 FP	CGGCGGCGTTTCGTGATCAATTT
45	StTm2 siR3 FP	CGGCGGTATCTTACTCGGCATA
46	StPHO2 -siR3 FP	GCGGCGGCCACCTAGAATATAAA
47	StPHO2 -siR4 FP	CGGGCTTGTTGTGGTCCTAGTAG
48	StHT4314-FP1	GAGTGCTATGTTGCTCGGGATCT
49	StHT4314-Rpi	GGTCAGATAGGGTGTGCTTTACGTTC
50	StTCP1253 FP1	GCGACCTAGTCCCAGTTGTGAC
51	StTCP1253 RPi	CCTTTCTGATCTGAATGCCGGTTGT
52	StPHO2 6435 TAS qFP	GGCACTTCTACTAGGACCACA
53	StPHO2 6435 TAS qRP	CGGTGGTGGAGTCTAACAACCT

2.3 Results

2.3.1 Small RNA expression profiles of stolon stages.

To identify small RNAs that are potentially involved during the stolon-to-tuber transition stages of potato in a photoperiod-dependent manner, we performed small RNA sequencing from stolon samples harvested on 4th, 7th, and 10th day of SD and LD induction (Fig. 2.1). Altogether, 188 million final clean reads were obtained from 12 small RNA libraries after adapter trimming and quality filtering. Of clean reads, 84.43% of reads were mapped to the potato genome. Approximately 12.68% of reads mapped to the known precursor miRNAs whereas 0.94% of reads were counted as a part of novel precursor miRNAs (Table 2.4). Small RNAs were divided into 21nt to 25nt category off which, 24nt long sequences were the most abundant in all libraries followed by 23, 22, and 21 nt sequences (Fig. 2.2).

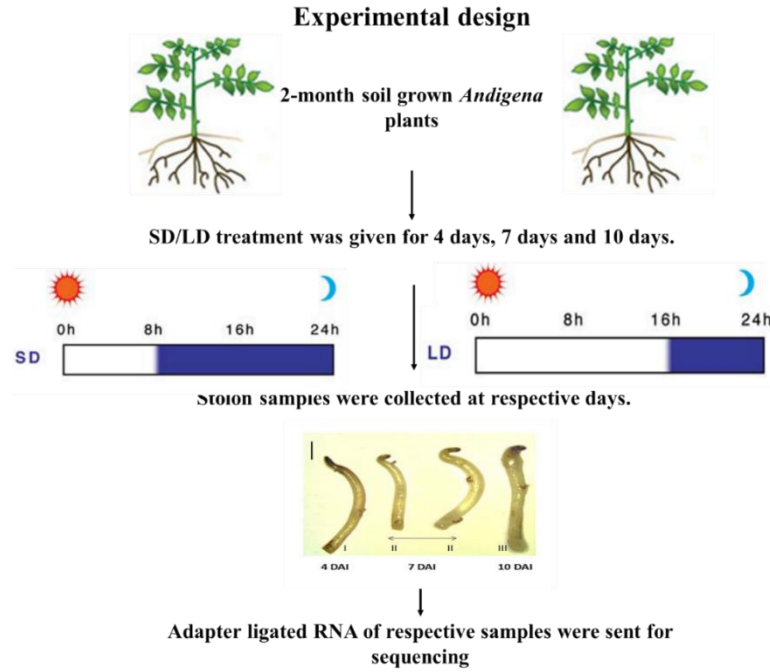


Fig. 2.1 Schematic representation for experimental design. Two month old soil-grown andigena plants were subjected to SD and LD treatment independently. Stolon samples were harvested on the 4th, 7th, and 10th day of incubation. RNA was isolated from samples and subjected to adapter ligation followed by the small RNA sequencing.

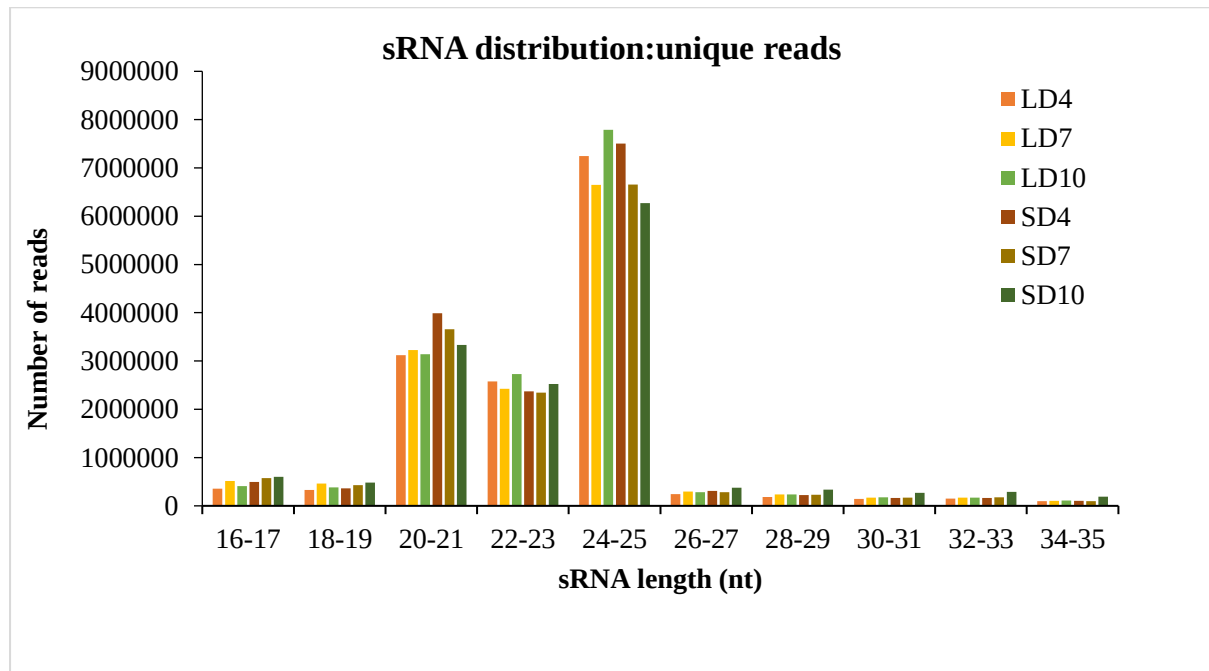


Fig. 2.2 Size distribution profile of small RNA sequences identified in sRNA libraries. Samples LD4, LD7, and LD10 represent 4, 7, and 10 days of long-day induction, whereas samples SD4, SD7, and SD10 represent 4, 7, and 10 days of short-day induction respectively.

Table 2.4. Summary of reads obtained from stolon small RNA libraries.

Types of reads/miRNAs	No. of reads/miRNAs
Good-quality reads after trimming	220,646,836
Final clean reads after adapter trimming	188,071,173
Final clean reads counted as conserved mature miRNAs	8,690,556
Conserved mature miRNAs in all samples	324
Conserved miRNA families in all samples	114
Final clean reads mapped to novel pre-miRNAs (hairpins)	1,759,128
Final clean reads counted as novel mature miRNAs	1,442,393
Novel mature miRNAs in all samples	311

2.3.2 Identification of conserved and potato specific miRNAs

The sRNA analysis with miRPro showed that on average 4.6% and 0.76% of final clean reads were counted as conserved and novel miRNAs, respectively. In our study, we detected 324 conserved and 311 novel miRNAs from stolons. Conserved miRNAs were obtained from 111 miRNA families (Table 2.4). Out of 311 novel miRNAs identified, star strand was detected in 270 miRNAs, with a total of 173 miRNAs having star strand present in at least two sequencing libraries. As the number of novel miRNAs predicted was higher than expected, we analyzed available small RNA sequencing datasets from potato leaves and stolons (Zhang et al., 2013), and identified a total of 152 novel miRNAs from these libraries (Fig. 2.3).

Published paper (Data accession number)	Kondhare et al., 2018 (SRP135981)	Data from Zhang et al., 2013 (GSE39230)
Samples and comparison	Stolon stages (4, 7 and 10 d) LD; 16 h light, 8 h dark and SD; 8 h light, 16 h dark	Leaves with SD and LD induction for 14 days, 12d stolons and 16d stolons
Number of conserved miRNAs	343	293
Number of novel miRNAs	311	152
Number of DE conserved miRNAs	09	Leaf (04) Stolon (30)
Number of DE novel miRNAs	08	Leaf (07) Stolons (11)

Fig. 2.3 Comparison of sRNA analysis between the small RNA sequencing datasets from the lab and previously available dataset.

2.3.3 Differential expression (DE) analysis of miRNAs

To identify photoperiod-induced miRNAs, differential expression analysis was carried out using DEseq2, and MA scatter plot showing Log2-fold change (y-axis) of conserved and novel microRNAs, for pairwise comparisons were made (Fig. 2.4). Similar analysis was carried out with the dataset from Zhang et al., 2013 (Fig. 2.5). Seven conserved miRNAs were differentially expressed between LD4 and SD4 conditions, of which two miRNAs were also differentially expressed in LD10 vs SD10 conditions (Table 2.5). For Novel miRNAs, 10 were differentially expressed under LD4 vs SD4 conditions, and two between LD10 and SD10 conditions. We did not observe any DE miRNAs for LD7 and SD7 comparisons (LD7 vs SD7) (Table 2.4).

DE miRNAs were validated by quantitative PCRs. We found that the expression data of RNA-seq and qPCR analysis was highly correlated (Kondhare et al., 2018). Further, heat maps were prepared for the top 30 representative conserved and novel miRNAs from 12 stolon small RNA libraries (Fig. 2.6).

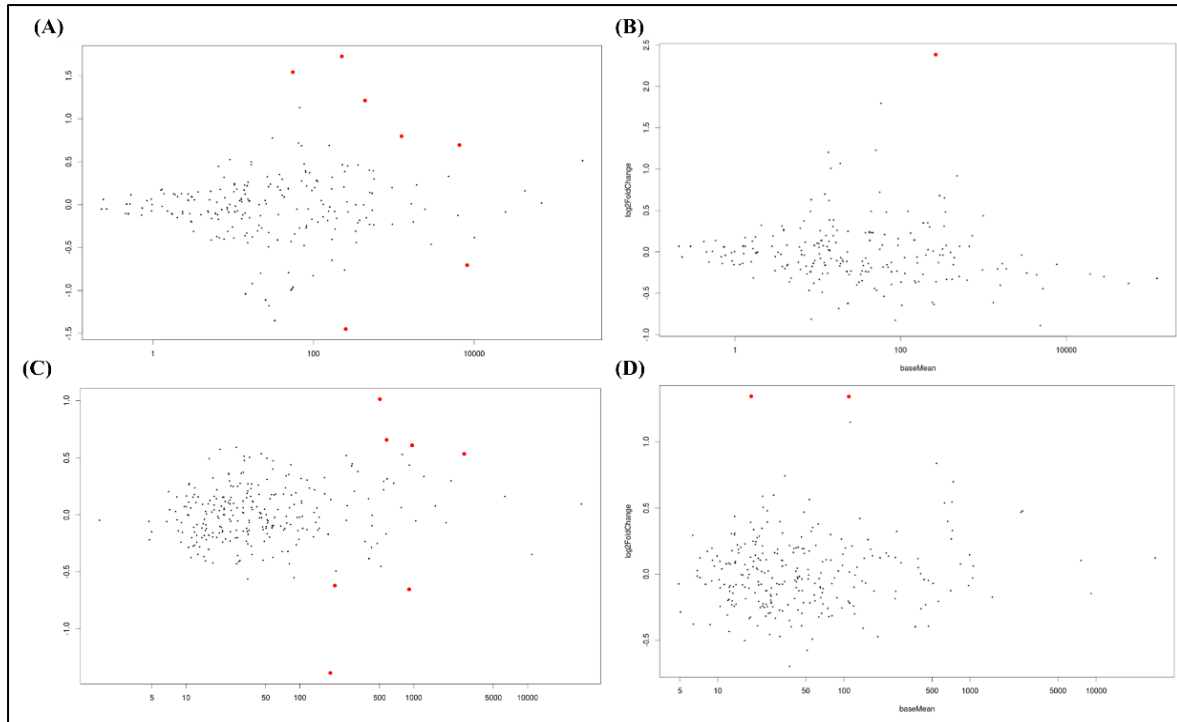


Fig. 2.4 MA scatter plot analysis for conserved and novel miRNAs. MA scatter plots showing Log2-fold change (y-axis) of pairwise comparisons for conserved (A and B) and novel (C and D) microRNAs, respectively. Conditions: LD4 vs SD4 (A and C) and LD10 vs SD10 (B and D). Differentially expressed miRNAs in each comparison are highlighted in red dots. BaseMean is represented on the x-axis.

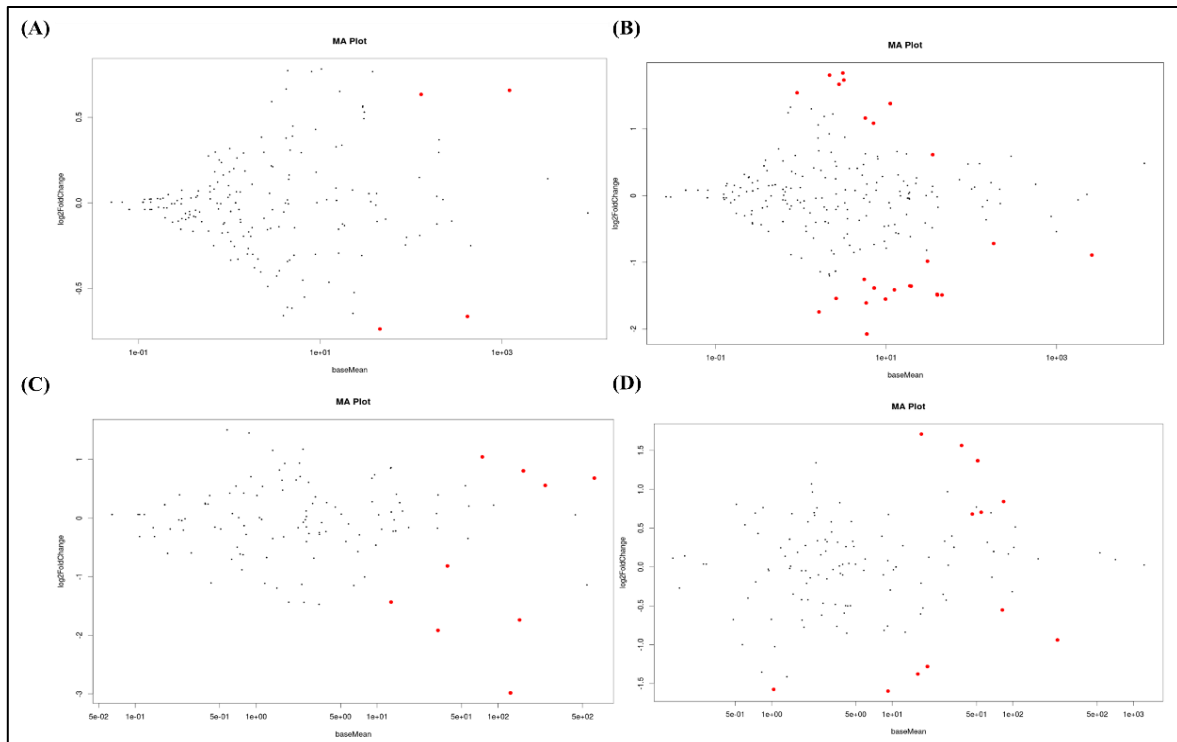


Fig. 2.5 MA scatter plot analysis for DE miRNAs from Zhang et al., 2013. Pairwise comparisons for conserved (A and B) and novel (C and D) microRNAs respectively, with LD4 vs SD4 (A and C), and LD10 vs SD10 (B and D) is represented. Differentially expressed miRNAs in each comparison are highlighted in red dots. BaseMean is represented on the x-axis and Log 2-fold change is shown on y-axis.

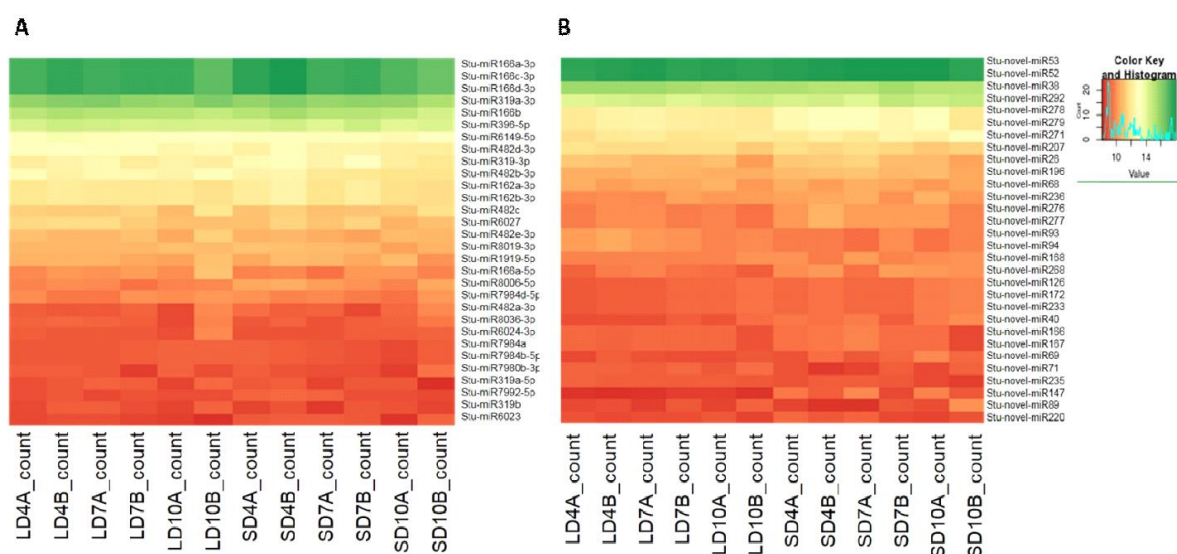


Fig. 2.6 Heat map clustering for (A) Conserved miRNAs and (B) novel miRNAs from stolon libraries.

Further, expression of DE miRNAs across the samples was analyzed through quantitative RT-PCR, and compared with the RNA-Seq Data (Fig. 2.7). Two conserved miRNAs (miR477a-5p and miR477b-5p) showed significantly higher expression at SD4 and SD10 time-points compared to the LD4, and miR477b-5p exhibited a reduction in its expression at LD10 compared to LD4 (Fig. 2.8 A-B). MiR319b-3p expression was significantly higher at SD4 compared to LD4, however, its expression did not change at other time points tested (Fig. 2.8 C). MiR482d-3p and miR399g-3p expressions were reduced under SD induction as compared to LD4 (Fig. 2.8 D-E). MiR479 exhibited highest expression in SD4 condition. However, their expression did not vary at other time-points (Fig. 2.8 F).

Table 2.5: List of differentially expressed conserved as well as novel miRNAs identified from LD4 vs SD4 and LD10 vs SD10 stolon transition comparisons. Number of putative targets predicted by psRNAtarget for each miRNA are represented.

	miRNA	log2FoldChange	p value	p adjusted value	No. of predicted targets (E<3.0)
Conserved miRNAs					
LD4 vs SD4	stu-miR399g-3p	-1.45	0.0006	0.0196	0
	stu-miR482d-3p	-0.71	0.0003	0.0101	18
	stu-miR319-3p	0.69	0.0001	0.0070	11
	stu-miR8006-5p	0.80	0.0001	0.0070	3
	stu-miR479	1.21	0.0000	0.0010	3
	stu-miR477b-5p	1.54	0.0008	0.0210	4
	stu-miR477a-5p	1.73	0.0000	0.0001	4
LD10 vs SD10	stu-miR477b-5p	1.79	0.0003	0.0526	4
	stu-miR477a-5p	2.38	0.0000	0.0001	4
Novel miRNAs					
LD4 vs SD4	stu-miR302	-1.39	0.0000	0.0000	6
	stu-miR93	-0.65	0.0002	0.0036	3
	stu-miR94	-0.65	0.0002	0.0036	3
	stu-miR221	-0.62	0.0055	0.0353	3
	stu-miR278	0.53	0.0041	0.0288	0
	stu-miR279	0.53	0.0041	0.0288	0
	stu-miR276	0.61	0.0022	0.0202	17
	stu-miR277	0.61	0.0022	0.0202	17
	stu-miR40	0.66	0.0003	0.0036	0
	stu-miR147	1.01	0.0000	0.0000	3
LD10 vs SD10	stu-miR139	1.34	0.0002	0.0289	0
	stu-miR206	1.34	0.0001	0.0289	4

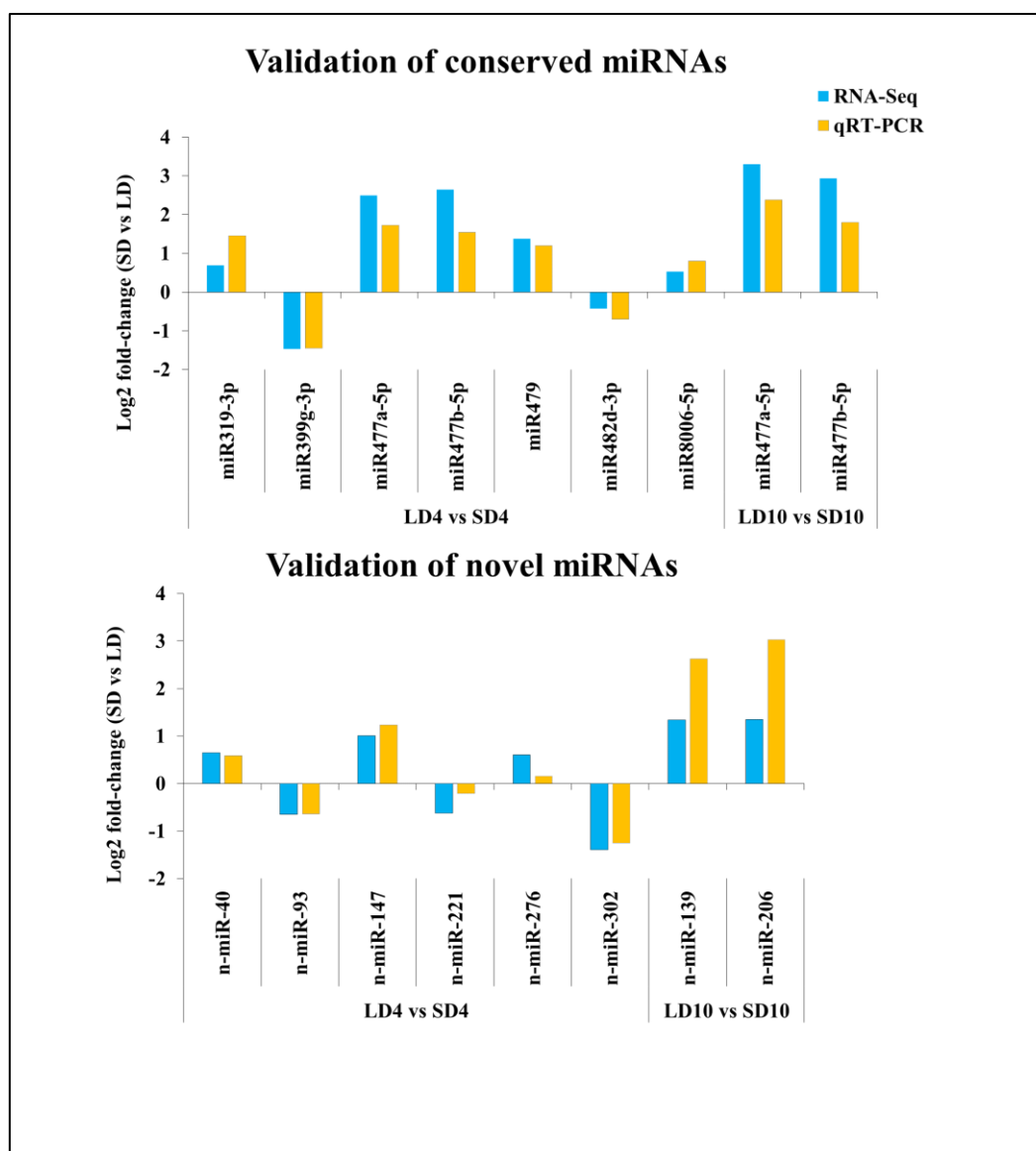


Fig. 2.7 Validation of sRNA sequencing data by quantitative PCR. Differentially expressed miRNAs were selected for analysis. (A) Validation of conserved miRNAs. (B) Validation of novel miRNAs. *StU6* was used as a reference gene for the normalization of qRT-PCR data. The expression of miRNAs under LD at the corresponding time point was considered as 1 to measure relative expression at respective SD time-points. Data is represented as means from two biological replicates and three technical replicates.

One novel miRNA (*n-miR-147*) showed a significant increase in its expression level under SD conditions compared to LD conditions at 4 and 7 d time-points (Fig. 2.9 A). The expression of the novel miRNA (*n-miR-206*) was significantly high under SD conditions than LD conditions at all three time points tested, but the difference was more distinct at 7 and 10 d than the 4 d time-point (Fig. 2.9 B). The *n-miR-139*, exhibited low expression at SD4 compared to LD4, but its expression significantly increased towards SD10 time point. Another novel

miRNA (n-miR-40) had significantly high expression at SD4 as well as LD7 and SD7 time points when compared with LD4 (Fig. 2.9 C-D). We found that n-miR-302 and n-miR-93 exhibited a distinct reduction in their expression levels at the 4 d time-point under SD compared compared to LD conditions (Fig. 2.9 E-F).

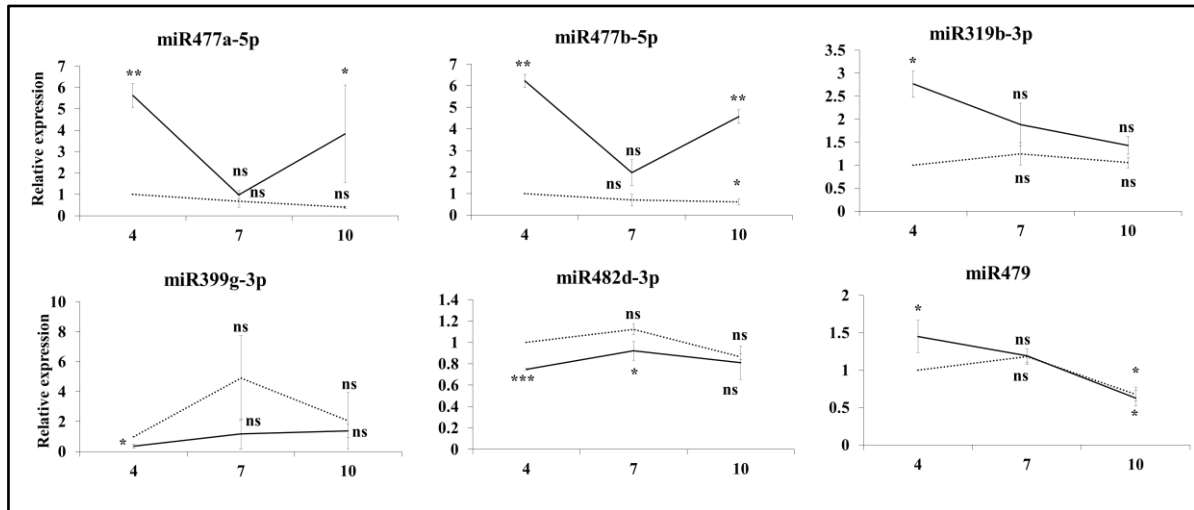


Fig. 2.8 Expression analysis of differentially expressed conserved miRNAs in stolons under LD vs SD photoperiod conditions. For qRT-PCR analysis, *StU6* was used as a reference gene for normalization, and the expression level of miRNAs at LD4 was considered as 1 for measurement of relative expression at other time-points. Error bars represent standard error of means (SEM) from two biological replicates. For graphs, a dotted line represents the LD condition, whereas a thick line represents the SD condition. Asterisks (one and two) indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively, using a Student's t-test. ns = not significant at $p < 0.05$.

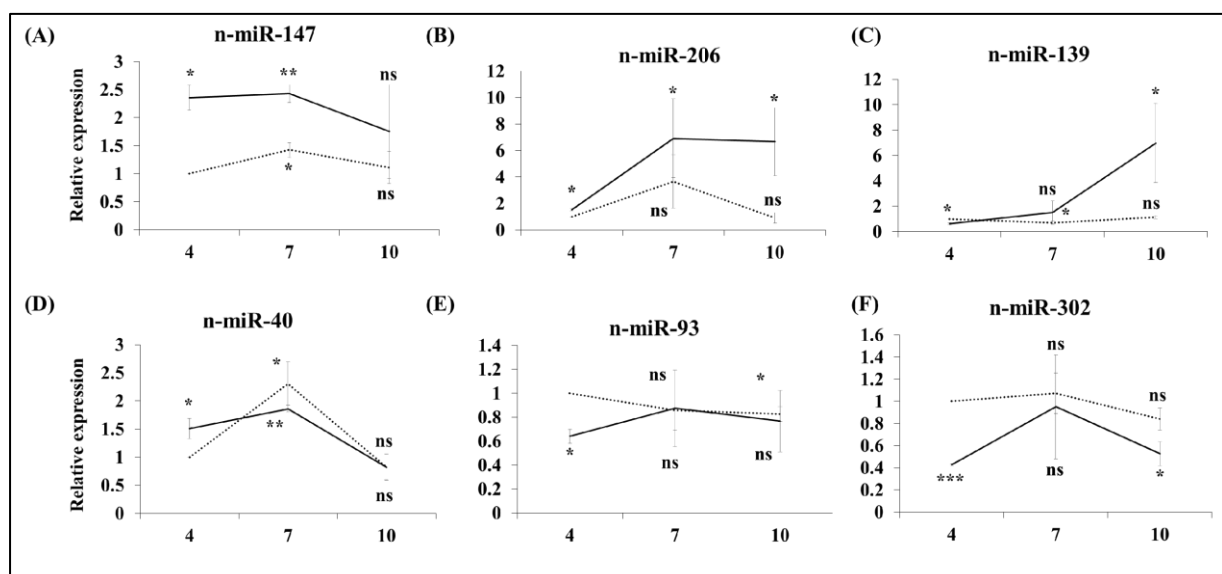


Fig. 2.9 Expression analysis of differentially expressed Novel miRNAs in stolons under LD vs SD photoperiod conditions. For qRT-PCR analysis, *StU6* was used as a reference gene for normalization and the expression level of miRNAs at LD4 was considered as 1 for measurement of relative expression at other time-points. Error bars represent standard error of means (SEM) from two biological replicates. For graphs, a dotted line represents the LD condition, whereas a thick line represents the SD condition. Asterisks (one and two) indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively, using a Student's t-test. ns = not significant at $p < 0.05$.

2.3.4 Target predictions and GO analysis

To explore the functional role of identified miRNAs, their putative targets were predicted with the help of psRNA target finder (plantgrn.noble.org/psRNATarget, Dai et al., 2011) software. Altogether 1414 putative targets were identified for 635 total miRNAs identified. For differentially expressed miRNAs, several interesting genes were identified, such as GRAS family transcription factors (TF) DELLA and SCARECROW (targeted by miR477a/b-5p and miR479, respectively), TEOSINTE BRANCHED 1, cycloidea and PCF TFs TCP2 and TCP4 (both targeted by miR319-3p), PROTEIN PHOSPHATASE 2c (targeted by miR8006-5p), CYTOCHROME P450 (targeted by novel-miR-147) and methyl transferase ASHR3 (targeted by novel-miR-221).

In our Gene Ontology (GO) analysis we found that, out of 1970 GO terms, 1235 GO terms belong to biological processes (BP), 236 GO terms were included under cellular components (CC), whereas 499 GO terms were categorized as molecular functions (MF) (Fig. 2.10). This categorization further revealed that auxin, cytokinin, and gibberellin (GA) metabolism genes were identified as targets of miRNAs. Example includes genes for auxin biosynthesis genes such as YUCCA8, auxin signaling gene TIR1-L (TRANSPORT INHIBITOR RESPONSE 1-like), auxin response factors (ARF10/16) as well as CK-related genes, i.e., cytokinin dehydrogenase, LOG10, zeatin O-glucosyltransferase, and UDP-glycosyltransferase. We found a GA precursor (GGPP) biosynthesis enzyme (farnesyl pyrophosphate synthase), and a GA signaling regulator (DELLA RGL1-like) as targets for these miRNAs. Light-mediated response related genes were also found to be the targets of miRNAs e.g. Cryptochrome 1, Cryptochrome-1-like isoform X1, ultraviolet-B receptor UVR8, transcription factor PHYTOCHROME INTERACTING FACTOR 1 (PIF1-like), B-box zinc finger 19-like (BBX19-L).

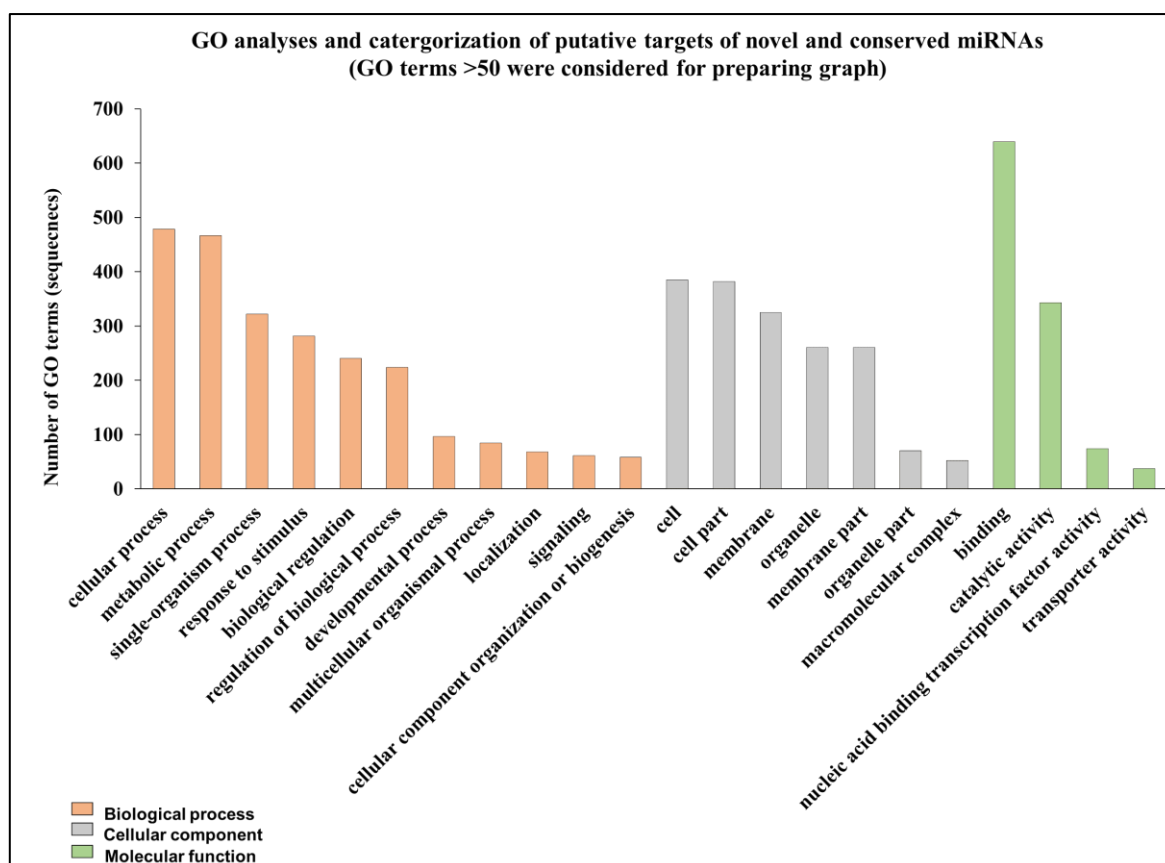


Fig. 2.10 Gene Ontology (GO) categorization for predicted targets of identified novel and conserved miRNAs. GO terms were categorized into biological process, cellular components, and molecular functions. GO terms with > 10 sequences were considered for preparing a graph in each category.

2.3.5 Time course expression analysis for miRNA-target pairs

The expression analysis of five miRNAs and their respective targets was carried out at the 4d time-point under SD and LD conditions. MiR477a-5p, miR319-3p, miR479 and n-miR-206 were up-regulated under SD as compared to LD photoperiod, whereas the expression for their respective targets (REPLICATION FACTOR C [miR377a-5p], StTCP2 [miR319-3p], StGRAS [miR479], EXOTOSIN FAMILY PROTEIN [n-miR-206]) were downregulated (Fig 2.11 A-C, E). N-miR-302 expression was reduced at SD4 time point whereas, the expression of its target, Metal dependent phosphohydrolase HD domain containing protein was upregulated (Fig 2.11 D).

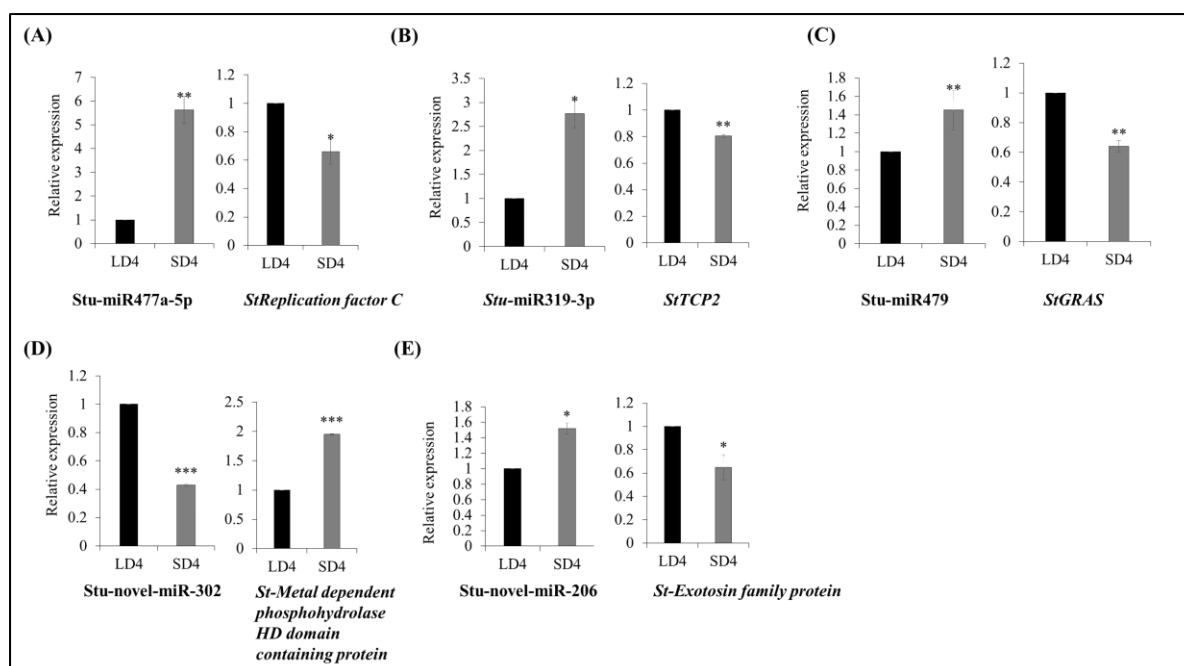


Fig. 2.11 Relationship between miRNAs and their predicted targets by qRT-PCR analysis at 4 d time-point under LD vs SD photoperiod conditions. Conserved miRNAs and their targets - (A) miR477a-5p and its target *replication factor C*, (B) miR319-3p and its targets *TCP* or *TCP4*, (C) miR479 and its target *GRAS* transcription factor. Novel miRNAs and their targets (D) n-miR302 and its target metal dependent phosphohydrolase HD domain containing protein, and (E) n-miR206 and its target exostosin family protein. *U6* and *EIF3e* were used for the normalization of miRNAs and their targets, respectively. The expression level of respective miRNA or target gene at the LD4 time point was considered as 1 to measure relative expression at the SD4 time point. Error bars represent the standard error of means from two biological replicates. Asterisks (one, two and three) indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, using a Student's t-test.

2.3.6 Cleavage site mapping for targets of conserved miRNA through 5' RLM-RACE

A modified 5' RLM-RACE analysis was performed to locate cleavage sites of miRNAs on its targets. *StARF10* was identified as the target of miR160 with high cleavage frequency (7 of 7) at 10th/11th nt position. Whereas, *StGRAS* (10 of 10) and *StGAMYB* (12 of 12) were found to be true targets of miR479 and miR319b, respectively (Fig. 2.12 A-C). *StTm2* transcript was cleaved by miR6026-3p with a very low frequency (1/11) (Fig 2.12 D).

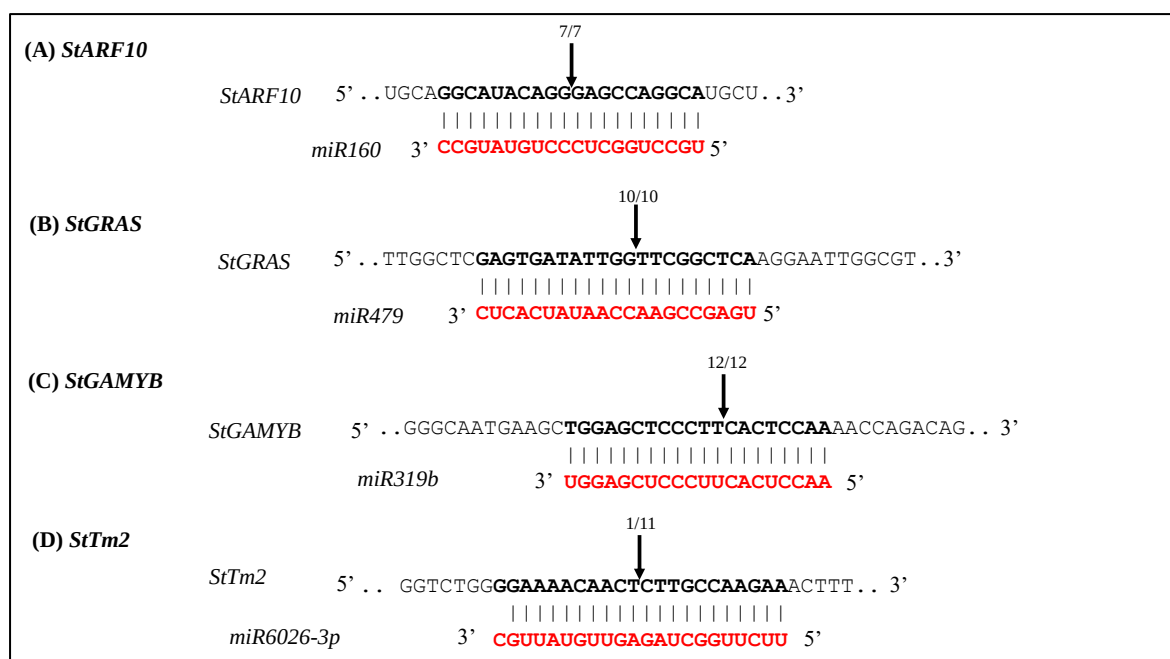


Fig. 2.12 5' RLM-RACE for cleavage site mapping. Arrows show frequency of 5' RACE clones showing cleavage sites and numbers represent fractions with proportions of clones showing these cleavage sites. *StARF10*, which is a known target of *Stu-miR160* (A) was used as a positive control as shown in Natarajan et al. (2018). Two conserved miRNAs (*stu-miR479* and *stu-miR319b*) were chosen to map cleavage sites on *StGRAS* (B) and *StGAMYB* (C) transcription factors, respectively. Cleavage site mapping for *miR6026-3p* is shown on *StTm2 TAS-like* locus (D).

2.3.7 Identification of phased siRNAs

We identified 830 putative phased siRNA-producing (*TAS-like*) loci from small RNA sequencing data. TASI-prediction tool from UEA sRNA workbench (V4.03) with stringent parameter of $p < 0.0001$ was used for the analysis (Fig. 2.13).

P value for Loci prediction	No. of <i>TAS-like</i> loci
0.0001	830
0.001	4442
0.01	23645
0.05	12652

Fig 2.13. The number of *TAS-like* loci identified using different parameters (*p*-values). *TAS-like* loci were predicted using the TASI-prediction tool with different *p* values and loci with stringent *p*-value (0.0001) were taken for further study.

From these loci, 275 loci were located in the genic region whereas, 555 were present in the intergenic region of the potato genome. Many of the genic region loci were found to be

residing within genes from growth development roles, such as auxin hydrogen symporter, PHO2, serine threonine protein kinase, TIR-1, Squamosa promoter binding, WD repeat protein, Cytochrome P450 like, AUX/IAA2, P450 monooxygenase, F-box family protein, and AGO1 like. Of 830 loci, 11 loci, and 13 loci were potentially targeted by conserved and novel miRNAs identified in this study (Table 2.5). As miRNA or phasiRNA-derived cleavage of *TAS* transcript is necessary for phasiRNA production, we considered *TAS-like* loci that are being targeted by miRNAs.

Table 2.6: List of *TAS-like* loci predicted to be cleaved by conserved or novel miRNAs. PGSC transcript IDs and gene annotation for *TAS* like loci that are present in the genic region is mentioned.

Sr. No.	<i>TAS-like</i> Locus	Genomic location	miRNA	Gene id	Gene annotation
1	Chr11:458098..458349	Intergenic	stu-miR5303g	PGSC0003DMT400034561	<i>Inositol monophosphatase 3</i>
2	Chr00:19041735..19041986	Intergenic	stu-miR8005a/b/c	None	None
3	Chr12:27420198..27420449	Intergenic	stu-miR8008a, stu-miR8009	None	None
4	Chr06:5520530..5520781	Exonic	stu-miR482c	PGSC0003DMT400022440	<i>NBS-coding resistance gene protein</i>
5	Chr02:33977738..33977989	Exonic	stu-miR399a-f	PGSC0003DMT400076435	<i>PHO2</i>
6	Chr00:14668823..14669074	Intergenic	stu-miR8005a/c; stu-miR8005b-3p	None	None
7	Chr00:39466733..39466984	Intergenic	stu-miR8005a/c; stu-miR8005b-3p	None	None
8	Chr00:41890891..41891142	Intergenic	stu-miR8005a/c; stu-miR8005b-3p	None	None
9	Chr09:15393324..15393575	Exonic	stu-miR6026-3p	PGSC0003DMT400051269	<i>Tm2</i>
10	Chr02:22364494..22364745	Intergenic	stu-miR7983-5p	None	<i>Hydroxyproline-rich glycoprotein</i>
11	Chr01:88411142..88411393	Intergenic	stu-miR5303a-f	None	Hypothetical gene of unknown function
12	Chr04:4747066..4747317	Intergenic	stu-novel-miR-124	None	None

13	Chr09:46314301..46314552	Exonic	stu-novel-miR-213	PGSC0003DMT400022135	<i>Disease resistance protein RGA4</i>
14	Chr10:45893671..45893922	Exonic	stu-novel-miR-263	PGSC0003DMT400068569	<i>Cc-nbs-lrr resistant protein</i>
15	Chr11:42040081..42040332	Intergenic	stu-novel-miR-272	None	None
16	Chr11:38087726..38087977	Exonic	stu-novel-miR-272	PGSC0003DMT400018940	<i>Disease resistance protein R3a</i>
17	Chr11:6031307..6031558	Intergenic	stu-novel-miR-276	None	None
18	Chr12:29516380..29516631	Exonic	stu-novel-miR-52, stu-novel-miR-53, stu-novel-miR-72	PGSC0003DMT400086126	Conserved gene of unknown function
19	Chr02:18433855..18434106	Intergenic	stu-novel-miR-71	None	None
20	Chr08:54903243..54903494	Intergenic	stu-novel-miR-171	None	None
21	Chr04:5836408..5836659	Intergenic	stu-novel-miR-124	None	None
22	Chr12:27420198..27420449	Intergenic	stu-novel-miR-13	None	None
23	Chr03:44097532..44097783	Exonic	stu-novel-miR-204	PGSC0003DMT400063143	<i>Formin 20</i>
24	Chr08:9967120..9967371	Intergenic	stu-novel-miR-41, stu-novel-miR-230	None	N

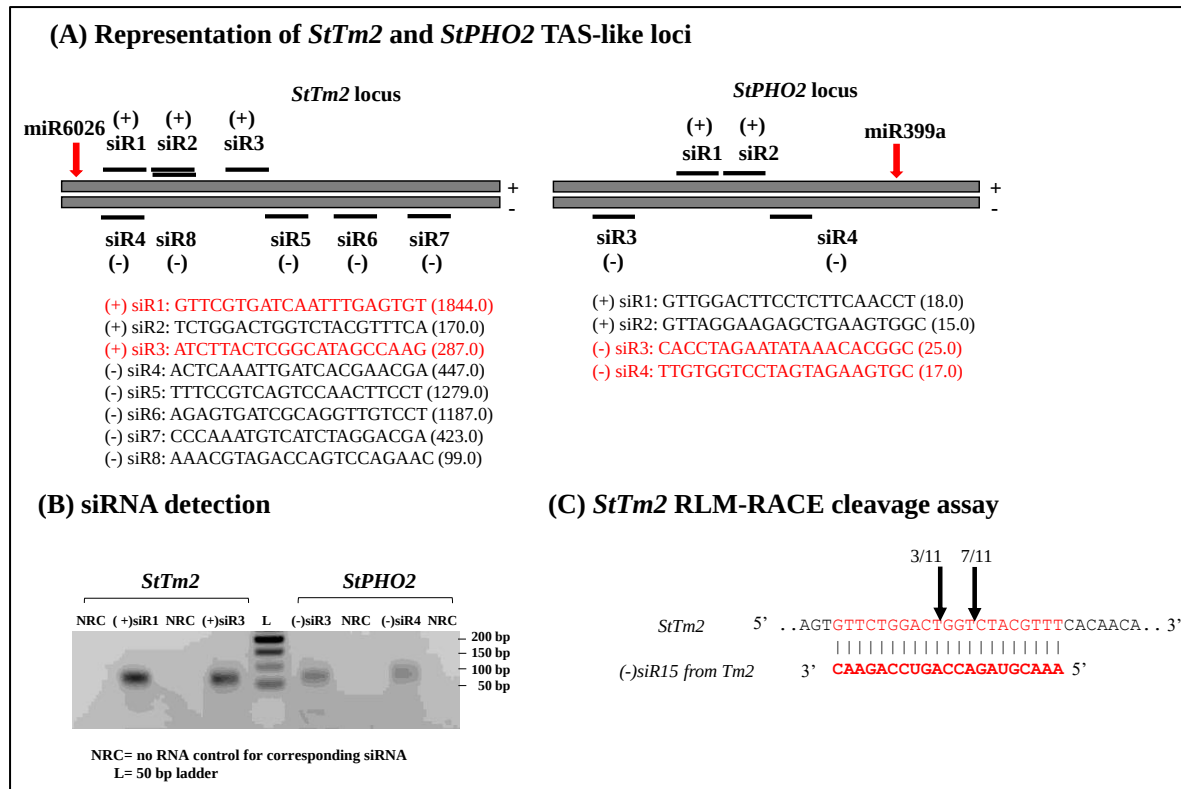


Fig. 2.14 Graphical representation of *StTm2* and *StPHO2*, two TAS-like loci and the most abundant siRNAs generated from these two loci are shown. (A) Phased siRNAs predicted to be generated from *StTm2* and *StPHO2*. Eight siRNAs off 18, whereas 4 siRNAs off 8, with higher abundance values are shown for both loci, respectively. Black arrows pointing downwards represent the predicted miRNA cleavage site in each locus. (B) Detection of two-phased siRNAs (highlighted in bold in panel (a) on the sense strand of *StTm2* ([+]siR1 and [+]siR3) and two from antisense strand of *StPHO2* ([-]siR3 and [-]siR4) by RT-PCR. (C) 5' RLM-RACE assay confirms the cleavage of *StTm2* by one of the siRNAs generated from itself i.e. (-)siR8. The siRNA cleavage frequency is also shown by downward arrows in black (10/11). Alignment between mature miRNA (bold) and target gene sequence is shown.

2.3.8 Validation of TAS like loci and putative phasiRNAs

We selected two putative TAS like loci Chr09:15393324..15393575 and Chr02:33977738..33977989, that are present within the genic regions of *StTm2* (predicted target of miR6026-3p) and *StPHO2* (predicted target of miR399a), respectively. *StTm2* and *StPHO2* were predicted to produce 18 and 8 phasiRNAs, respectively (Fig 2.14 A). Subsequently, two phasiRNAs generated from each locus were detected in stolon samples and sequence confirmed (Fig 2.14 B). We were not able to confirm *StPHO2* as a target of miR399a through 5' RLM-RACE. However, *StTm2* was shown to cleave by miR6026-3p at a very low

frequency 1/11. Interestingly, we observed, one of the siRNAs generated from the *StTm2* locus, i.e., siR8(-), cleaves its own locus with 10 of 11 cleavage frequency that might generate a pool of secondary siRNAs from cleaved transcript (Fig. 2.14 C). Since the cleavage site of miR6026-3p on the *StTm2* locus is only 65 bp upstream to siR8(-) cleavage site, this could be the reason why the cleavage frequency by miR6026 was very low (1/11) on this locus. It also confirms that siR8(-) functions as a phasiRNA targeting the loci from which it is being generated.

We performed expression analysis of the *StTm2* and *StPHO2* as well as siRNAs in stolon samples (Kondhare et al., 2018), and observed that expression of *StTm2* remains low at early stolon stages and it increases at the SD10 time point whereas siR1(+) and siR3(+) expression remain high at SD4 and SD7, and decreases at SD10. *StPHO2* expression was reduced at SD7 stage and then remained high at later SD induced stolon stages. siR3(-) and siR4(-) showed inverse expression as compared to *StPHO2* at SD7 and SD10 stolon stages.

2.3.9 Conserved TAS loci in potato

Our analysis identified on *TAS* locus Chr01:37276576..37276827 that fulfilled criteria to be presented as *TAS3* locus. This locus was present in the genic region of a hypothetical protein (PGSC0003DMT400034044), and contains two miR390 cleavage sites. *StTAS3* transcript was aligned to *AtTAS3b*, *NtTAS3a* (1) and *NtTAS3a* (2), and *SITAS3*, showing close conservation within *TAS3* locus from the plant species (Fig 2.15 A). *StTAS3-siR1* (abundance value 134) and *StTAS3-siR2* (abundance value 148) are conserved in all plant species (Fig 2.15 B). These siRNAs were predicted to cleave *StARF3* and *StARF2* transcripts with expectancy values of 0.5 and 1.0, respectively (Fig 2.15 C).

Apart from *StTAS3*, we also observed the *TAS5* locus (Chr06:5518510..5518761) from our sequencing data, residing within the NBS-coding resistance gene (PGSC0003DMT400022440). This locus shared a close conservation with *SITAS5* from tomato (Fig 2.15 D). Moreover, the target site of miR482c/miR482a-3p on these loci was conserved. Similarly, *StTAS5-siRNA*(-), with abundance value of 1734, displayed a high conservation with a tomato *SITAS5 -3'DI2*(-) (Fig 2.15 E).

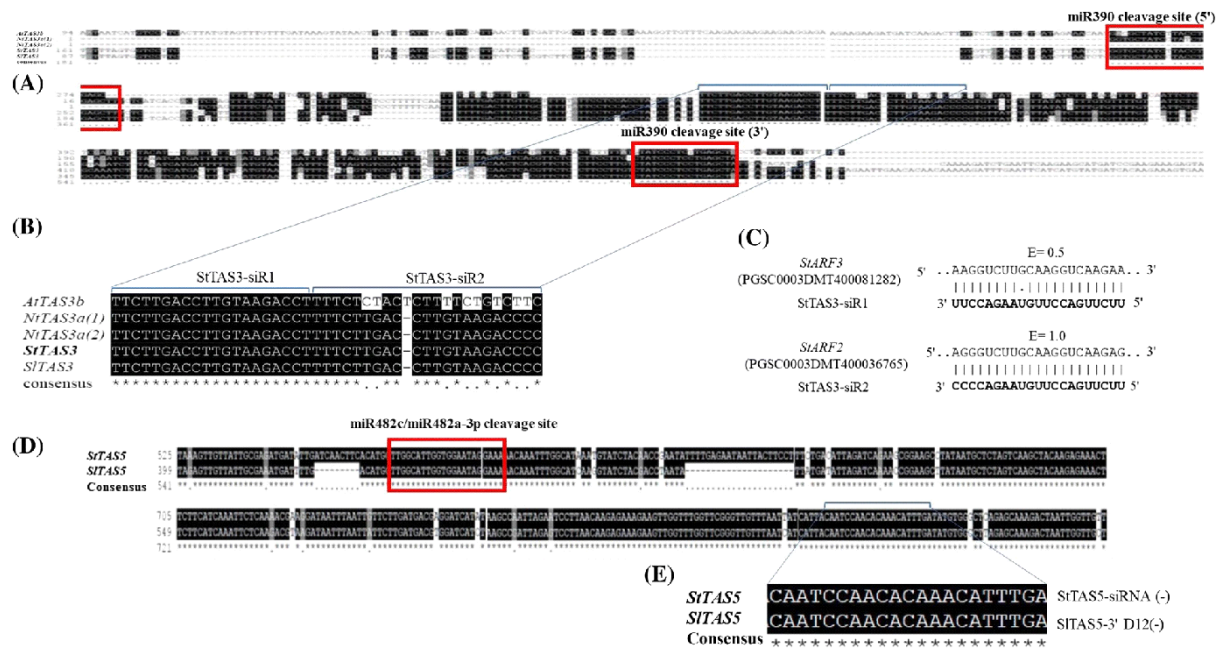


Figure 2.15 Alignment of TAS3 and TAS5 genes from Solanaceae family. (A) Alignment for a TAS3 region from TAS3 transcripts Arabidopsis thaliana *AtTAS3b*, *Nicotiana tabacum NtTAS3a* (1) and *NtTAS3a* (2), *Solanum tuberosum StTAS3*, and *Solanum lycopersicum SITAS3*. MiR390 target sites (5' and 3') on all TAS3 transcripts are highlighted with red boxes. (B) Magnified view of the alignment of TAS3-derived siRNAs from above mentioned plant species. *StTAS3-siR1* and *StTAS3-siR2* showed a close conservation across chosen plant species. (C) psRNA target prediction represents *StTAS3-siR1* cleaves auxin response factor *StARF3*, whereas *StTAS3-siR2* cleaves *StARF2* with a cleavage expectancy of $E=0.5$ and $E=1.0$, respectively. (D) Alignment of *Solanum tuberosum StTAS5* transcript with that of *Solanum lycopersicum SITAS5* transcript. MiR482c/miR482a-3p target site is highlighted in the red box. (E) Magnified view of aligned TAS5 transcripts from potato and tomato. *StTAS5-siRNA* (-) exhibits high conservation with tomato *SITAS5-3'D12*(-).

2.3.10 Putative phasiRNAs that could regulate key tuberization genes

We identified 59 phased siRNAs generated from 11 TAS-like loci targeted by conserved miRNAs whereas, 131 phased siRNAs were generated from 13 TAS-like loci targeted by novel miRNAs. Altogether, 3441 targets were identified as putative targets for these phasiRNAs. The GO analysis of targets showed that of 5918 GO terms, 1780 belong to biological processes, 2210 terms categorized in the molecular function category, whereas 1919 GO terms are included in cellular components (Kondhare et al., 2018). The siRNA target genes were highly enriched in starch and amino acid metabolism genes. Many of the targets were involved in transport, signaling and metabolism genes of hormones like auxin, cytokinin, ABA, gibberellin, and ethylene (Kondhare et al., 2018). We found that they target cell cycle and cell

division associated genes as well as key tuberization genes, such as homeobox TFs, F-box proteins, Early Flowering 3, Dof zinc finger protein (*StCDF4*), *POTH1*, phloem mobile RNA binding protein (*StPTB1*), and zinc/ring finger proteins. Interestingly, two of the phasiRNAs from *StTm2* and *StRGA4* were found to target key GA metabolic genes *StGA2ox1* and *StGA3ox*, respectively.

2.4 Discussion

2.4.1 Photoperiod influences the expression of miRNAs during stolon-to-tuber developmental transitions stages

Stolon acts as a center for coordinated action of TFs, mobile signals, and phytohormones during tuber development. As a result, the stolon undergoes developmental transitions wherein, the plane of cell division changes from longitudinal to radial at the sub-apical region of the stolon tip resulting in the swelling of the stolon, followed by accumulation of starch to form a mature tuber (Roumeliotis et al., 2012; Xu et al., 1998). The mechanistic basis for this regulation is still unclear.

As small RNAs have been shown to function as key drivers of various plant developmental processes by governing the gene regulatory networks, we speculate that the mechanism of early stolon-to-tuber development could also be controlled by TFs and small RNAs. In addition, small RNAs might help in fine-tuning the tuber formation process. Earlier, a few miRNAs such as *miR172*, *miR156*, and *miR390* have been demonstrated to regulate tuber development (Martin et al., 2009; Bhogale et al., 2014; Santin et al., 2017). However, their role in the stolon-to-tuber transition was unclear. Lakhotia et al (2014) identified a diverse population of miRNAs from early stolon transition stages. However, the study did not consider the effect of photoperiod on tuber formation.

Photoperiod is a critical factor that influences tuber development in potato. Day length affects miRNA expressions in other plant systems as well. For example, *miR163* has been shown to be induced by red/blue/white light whereas its target *AtPXMT1* (methyl transferase) was down-regulated during seedling de-etiolation and germination in *Arabidopsis* (Chung et al., 2016). Wu et al (2013) demonstrated that the expression of *pooideae*-specific *miR5200* reduced under long days compared to short days. It targets *FLOWERING LOCUS T* to govern floral transitions in *Brachypodium* (McKeown et al., 2017). To determine the influence of photoperiod on small RNA population in potato, we performed a sRNA profiling from stolons of a photosensitive cultivar (andigena) at three early stolon stages (4, 7, and 10 d) under LD

(non-inductive) versus SD (inductive) photoperiod conditions. SD4 and SD10 were used to represent different stages of stolon and tuber development, with SD4 representing the early stages and SD10 representing the late swollen stages. In this study, it was observed that there was a mixed stolon population at the SD7 d time-point. In our study, we identified 7 conserved and 12 novel miRNAs differentially expressed under SD at either 4d or 10d when compared with respective LD conditions. Our study indicates that photoperiod has a critical effect on miRNA expressions in early stolon stages of tuber development. Since 17 out of 19 differentially expressed miRNAs (~ 90%), were found at LD4 vs SD4 comparison (Fig. 2.4; 2.7). We also identified 6 miRNAs that are differentially regulated in SD10 as compared to the SD4 time point, suggesting varying expression of miRNAs in stolon-to-tuber development stages. This observation suggests that miRNAs might also be involved in regulating genes that coordinate stolon-to-tuber transition stages. Previously, the expression of miR156 and miR172 was shown to be induced in stolons at the 14 d time point under SD conditions. To our surprise, miR156 and miR172 did not show differential regulation at early time points implicating their role in tuberization, instead of being involved in early stolon transition stages.

2.4.2 DE miRNA and their targets exhibits potential role during tuber development

We identified several hormone metabolism and signaling-related genes as targets of DE conserved and novel miRNAs. Many of these targets exhibited a differential expression under LD4 vs SD4 photoperiod. GAMYB and DELLA (Carrera et al., 2012) TFs have an established role in GA-dependent signaling to promote flowering (Zhang et al., 2020). *StGRAS* was found to be targeted by miR479 in stolons. DNA binding activity of these TFs enables their function in root radial patterning and growth, which indicated that *miR479-StGRAS* (miRNA-target) pair might be involved in cell division patterns in stolons. TCPs have been well demonstrated to regulate the biosynthesis of various phytohormones, floral symmetry, plant architecture and lateral organ development, circadian rhythms, and defense responses (Li, 2015). Particularly, TCP2 functions as a transcriptional activator down-stream to the CRYPTOCHROME 1 (CRY1), photo-sensory signaling (He et al., 2016), whereas TCP4 play a major role in cell number maintenance and cell proliferation during leaf development. Inverse expression patterns of miR319-3p and its targets *StTCP2/4* under LD4 vs SD4 conditions indicated the putative role of *miR319-StTCP2/4* pair governing cell divisions in stolons.

The polypyrimidine tract-binding proteins in potato (*StPTB1* and 6) bind to *StBEL5* mRNA to facilitate transport of its mRNA from leaves to stolons under tuber inductive

conditions (Cho et al., 2015). StPTBs therefore, function as positive regulators of tuberization by enhancing *StBEL5* mRNA levels in stolons. Our target prediction analysis suggests that *StPTB6* mRNA could be the target of *miR156e*. Its levels increased under SD time-points suggesting a new mode of regulation of *StPTB6* via *miR156e* during tuberization. StBEL11 and StBEL29 are two close homologs of StBEL5 in potato. These phloem mobile signals unlike StBEL5, act as repressors of tuber yield (Ghate et al., 2017). Degradome analysis from cold-stored potato sprouts revealed *StBEL29* as a target of conserved miRNA (*miR172h*) (Ou et al., 2015). Novel miRNAs such as n-miR-221 and n-miR-230, predicted to target histone-lysine N-methyltransferases (*ASHR3* and *ATX4-like*, respectively), indicating epigenetic regulation of stolon development. Overall, our results suggest that miRNA-mediated regulation of target genes might be crucial to fine-tune the early stages of stolon-to-tuber transitions.

2.4.3 PhasiRNAs: A new paradigm to gene regulatory pathways involved in tuber development.

Plant phasiRNAs are known or are predicted to be involved in diverse biological processes. Given the large number of *PHAS* loci and populations of phasiRNAs predicted, their precise functions in plant development are still limited. A subset of phasiRNA, i.e. trans-acting siRNAs (tasiRNAs), are known to target complementary mRNAs and play major roles in plant development and stress responses (Liu et al., 2020; Dong et al., 2022). Previous small RNA transcriptome studies have not reported phased siRNAs from stolon-to-tuber stages of potato development. Only a few studies have predicted the repertoires of phased siRNAs from the *Solanaceae* family and the majority of them were shown to be involved in defense responses. For instance, the miR482/miR2118 family, initiates the production of phasiRNAs from specific disease resistance target genes. This process serves to modulate the expression of genes belonging to the NB-LRR family, which are known to play a crucial role in plant immunity against various pathogens (Zhai et al., 2011; Zhang et al., 2022). In this study, using the TA-SI prediction tool, we have identified 830 putative *TAS-like* loci from the potato genome, which can give rise to either phasi or tasiRNAs. We also found two conserved *TAS* loci (*StTAS3* and *StTAS5*) in our analysis. Finally, we analyzed targets of 190 phased siRNAs that are generated from 24 *TAS-like* loci by the action of miRNAs identified in our study. An in-depth analysis of siRNA-target pairs was carried out and based on their functions, siRNA targets were grouped into different categories like sugar metabolism/transport, flowering and photoperiod response, cell division and cell cycle, epigenetic modifiers, calcium signaling, and storage proteins. Interestingly, hormone metabolism, signaling, and transport pathway-related genes (auxin, GA,

CK, brassinosteroid, ethylene, and ABA) were identified as phasiRNA targets. Several key tuberization pathway genes, such as *StBEL5*, *StCO1/2*, *StCDF1*, *StGA2OX1*, *StGA3ox3*, and *StSUT1* were also identified as siRNAs targets (Table 2.7). In a previous study, phasiRNA931 was identified as having the potential to cleave a gene involved in GA biosynthesis in potato. It was observed that the expression of phasiRNA931 increased following infection, which coincided with a decrease in the expression of the GA biosynthesis gene. Moreover, it has been suggested by authors that the presence of a complex small RNA regulatory network in plants may contribute to establishing a balance between developmental processes and defense mechanisms. (Křižník et al., 2017). GA metabolism genes such as *StGA2ox1* and *StGA3ox3* have been validated by a modified 5'-RLM RACE suggesting that phasiRNA-mediated regulation of GA metabolism genes might be involved in tuber development. Altogether, our analysis indicated that phasiRNAs have the potential to regulate the events during stolon-to-tuber developmental transitions by targeting key tuberization genes.

To summarize, in this study, we carried out small RNA profiling of early stolon stages (4th, 7th, and 10th d) under SD/LD photoperiodic conditions, and identified differentially expressed 7 conserved and 12 novel miRNAs. Selective miRNA-target genes, also exhibited peculiar patterns across stolon stages such as miR479-*StGRAS* and miR319b-*StGAMYB*, indicating a putative role of miRNA-target pairs during tuber development. Further, we extended our analysis by identifying putative phasiRNAs producing *TAS-like* loci from stolons. We noted that 24 loci with miRNA triggers could generate 190 phasiRNAs. These phasiRNAs were found to target key tuberization genes, particularly photoperiod and hormone metabolism/signaling genes. Subsequently, 5' RLM-RACE assay confirmed GA biosynthesis gene (*StGA3ox*) as a target of a phasiRNA generated from an exonic region of a disease resistance gene (*StRGA4*). As regulation of gibberellin is essential during tuber development, it would be interesting to unravel the phasiRNA-mediated regulation of GA biosynthesis in stolon-to-tuber development. In the following chapter, we have discussed our findings regarding the role of a key phasiRNA, siRD29(-) in the regulation of a GA biosynthetic gene, *StGA3ox3*.

Table 2.7. List of miRNAs targeting putative *TAS-like* loci for generation of siRNAs that could target key tuberization genes. (Adapted from Kondhare et al., 2021; review)

TAS locus	miRNA cleaving TAS locus	siRNAs	siRNA abundance	PGSC transcript IDs	Genes
Ch04:4639310..4639561	stu-miR482d-3p	CTTGTGGTGGTTGATGATGTA	10	DMT40002606 4/68/69	StCO1
Ch04:4676138..4676389					
Ch04:5636455..5636706					
Ch04:4726792..4727043					
Ch04:5835885..5836136	stu-novel-miR-276	CATCCTCCATTCTTTCTTCTC	27		
Ch04:5637097..5637348					
Ch04:5735568..5735819					
Ch04:6052871..6053122					
Ch04:72108862..72109113	stu-novel-miR-194	AATCCGTCTGTGACCCGCCCT	19	DMT40002606 5/67	StCO2
		AGAAATCCGTCTGTGACCCGC	32		
Ch04:4639310..4639561	stu-miR482d-3p	CTTGTGGTGGTTGATGATGTA	10		
Ch04:4676138..4676389	stu-novel-miR-276				
Ch04:5636455..5636706					
Ch04:4726792..4727043					
Ch04:5835885..5836136					
Ch05:44635418..44635669	stu-miR7984a; stu-miR7984c-5p	TTTGGTATCGTAACAACAATT	2	DMT40004737 0	StCDF1
Ch05:1058327..1058578	stu-miR6023	ATGAATTAGTGAGTAGAATAG	7	DMT40005141 6	StFKF1
Ch05:4229419..4229670	stu-miR482d-3p; stu-novel-miR-276	ATGATCTTTGGAGTACTGATG	3	DMT40000939 3	StFD1
Ch01:48008140..48008391	stu-novel-miR-90	TACCATATAAGGCCTCGAATG	5	DMT40004172 6	StSP5G
Ch11:26140759..26141010	stu-miR171b-5p	TTCATATTCTTGTTTAGGGGG	13	DMT40004846 4/65	StPTB1
				DMT40006082 7/28	StPTB6
Ch10:48198808..48199059	stu-miR482d-3p; stu-novel-miR-286	TGAAGTCCGTTGTTGACTCA	43	DMT40004578 2	St14-3-3
Ch11:5777631..5777882	stu-miR482d-3p	TCTCTTCAATTACTGGAAGCA	2	DMT40001518 3	StBEL5
Ch08:54903243..54903494	stu-novel-miR-171	AAAACAAAGTTGAGTGACTTT	2	DMT40003509 5	POTH1
Ch11:6031307..6031558	stu-novel-miR-276	CTTTTATTGTCATCATTTGGA	22	DMT40008449 0	StE(z)2
Ch04:5637097..5637348	stu-novel-miR-124	CCAAGCTTCGTCTTATCAGCT	77	DMT40005434 8	StGA2OX1
Ch04:5735568..5735819					

Ch04:605287 1..6053122		CCAAGCTTCGCTCTTATCAGTT	179		
Ch09:463143 01..46314552	Stu-miR-482c	AATAGAACTTTCATCTTGAGG	10	DMT40007474 4	StGA3ox3
Ch02:357661 88..35766439	stu-miR8014-3p	GAGAGAGAGATTTGATATACA	2	DMT40006231 4	StGA20OX1
Ch01:470623 26..47062577	stu-miR482a-3p	TGAAAGATCAAATTTTAGACT	5	DMT40004803 4	StARF8
Ch12:220483 67..22048618	stu-miR7990a	TTTTGGAAAGAATGAAAAATC	2	DMT40001475 2	StPIN1
Ch12:644943 6..6449687	stu-miR390-5p; stu- novel-miR-225	ATTCACAAAACTGAAGGAT		DMT40007833 0/31	StPIN4
Ch02:176603 37..17660588	stu-miR7990a	TTTTGTAATTGTTTAAGGGCA	389	DMT40002380 4	SUCROSE TRANSPOR TER 1
Ch04:187065. .187316	stu-miR482a-3p	AAAAAAGAAAGGTTGTTTAAA	62	DMT40002380 4	(StSUT1)
Ch01:339628 6..3396537	stu-novel-miR-193	GTGCAAACAATTTCTGAGGGC	562	DMT40003526 0/61/62/63	SUCROSE SYNTHASE (StSUSY1)
Ch02:280300 70..28030321	stu-novel-miR-102; stu-novel-miR-127				
Ch03:151840 09..15184260	stu-novel-miR-126; stu-novel-miR-300				
Ch04:306173 41..30617592	stu-miR7997a				
Ch05:305977 91..30598042	stu-miR7981-3p				
Ch05:351841 06..35184357	stu-novel-miR-127; stu-novel-miR-102				
Ch08:497140 63..49714314	stu-novel-miR-100				
Ch09:610817 35..61081986	stu-novel-miR-108; stu-novel-miR-112				
Ch11:866546 2..8665713	stu-miR8048-5p				
Ch12:955262 5..9552876	stu-miR8022; stu- novel-miR-15				
Ch11:412351 95..41235446	stu-miR482e-3p	ATACAAAGACACCAGAATGCT	5	DMT40001108 1	StSWEET12 D
		TGGTTGGATGAACCTTCTCAAC	6		
Ch09:608537 83..60854034	stu-miR6027	TGCTGCCAGGTTTCATGATGTT	4	DMT40005357 2	CELLULOS E SYNTHASE (StCesA2)
Ch09:608769 03..60877154					
Ch09:608922 71..60892522					
Ch09:609045 75..60904826					

Part of this work has been published in the journal of BMC Plant Biology, Vol. 18, 284

Kondhare KR, **Malankar N**, Devani RS, Banerjee AK (2018) Genome-wide transcriptome analysis reveals small RNA profiles involved in early stages of stolon-to-tuber transitions in potato under photoperiodic conditions. **DOI:** 10.1186/s12870-018-1501-4.

Chapter 3

PhasiRNA siRD29(-) regulates a gibberellin biosynthesis gene *StGA3ox3* during stolon-to-tuber transitions in potato

3.1 Introduction

Small non-coding RNAs (sRNAs) control various aspects of plant growth and development (Liu et al., 2020; Dong et al., 2022). MicroRNAs (miRNAs) and phased secondary short interfering RNAs (phasiRNAs) constitute a major category of sRNAs and regulate the expression of target genes *via* transcriptional or post-transcriptional gene silencing (Fei et al., 2013). MiRNAs are 21-24 nt sRNAs, which form a RNA-induced silencing complex (RISC) with ARGONAUTE 1 (AGO1) to target mRNAs (Rogers and Chen, 2013; Borges and Martienssen, 2015). AGO1-RISC complex loaded with 22 nt miRNAs can trigger the biogenesis of sRNAs from target mRNAs. With the help of SUPPRESSOR OF GENE SILENCING (SGS3) and RNA-DEPENDENT RNA POLYMERASE6 (RDR6), cleaved mRNAs form dsRNAs (Yoshikawa et al., 2005). The resulting dsRNAs are then cleaved by a DICER-LIKE 4 protein (DCL4), forming phasiRNA duplexes that are distinctive and generated in phased configuration (Allen et al., 2005; Chen et al., 2010). The loci generating phased siRNAs are called as *PHAS* loci.

Although numerous *PHAS* loci have been identified from diverse plant species, only selective phasiRNAs are characterized for their functions in plant development. A subset of phasiRNAs, known as trans-acting siRNAs (tasiRNAs), are involved in seed germination, leaf and root development, shoot apical meristem (SAM) initiation, flowering time and pre-meiotic reproductive development (Liu et al., 2020). Major *TAS* loci members are conserved in plants. For example, miRNA390-*TAS3*-mediated regulation of *ARF* (*AUXIN RESPONSE FACTOR*) pathway is conserved across the plant lineages and controls developmental timing and patterning, leaf morphogenesis, and somatic embryogenesis (Xia et al., 2017). MiR166 and tasiRNAs targeting *ARFs* regulate initiation of shoot meristem and leaf polarity in maize and rice (Nagasaki et al., 2007; Nogueira et al., 2007). Another relatively conserved phasiRNA pathway is miR828 targeting *TAS4* or *MYB* transcription factors (TFs) (Xia et al., 2012) that are involved in biosynthesis of anthocyanin, flavonoids, lignin (Bonar et al., 2018; Tirumalai et al., 2019), and fruit development (Rock, 2013). Non-conserved phasiRNA pathways, such as miR3954-siRNAs-NAC TFs affects flowering time in citrus (*Citrus sinensis*) (Liu et al., 2017; Ma et al., 2018). A recent study showed that miR9678, a wheat specific miRNA targets lncRNA (WSGAR) to produce phasiRNAs, which regulate germination process *via* the altered response of two phytohormones, abscisic acid and gibberellin (GA) (Guo et al., 2018). However, siRNA targets in this pathway are unknown.

Tuber development mechanism (tuberization) in potato is a complex process governed by a coordinated action of various environmental, biochemical and genetic factors (Hannapel et al., 2017; Kondhare et al., 2020). During tuberization, a belowground modified stem (stolon) passes through different developmental transitions starting from swelling at the sub-apical region to the development of a mini-tuber, before maturing to a potato. At the onset of tuber formation, a dynamic change occurs in GA and auxin metabolism and their signaling pathways in the stolon. Several genes associated with auxin biosynthesis (*StYUC-LIKE1*), transport (*StPIN2* and *-4*), and signaling pathways (*ARFs* and *AUX/IAAs*) show differential expression during stolon-to-tuber transition stages (Kloosterman et al., 2008; Roumeliotis et al., 2012b; 2013b). Moreover, the expression of GA catabolism gene, *StGA2ox1* increases (Kloosterman et al., 2007), whereas the expression of GA biosynthesis genes (*StGA20ox1* and *StGA3ox2*) decreases under tuber-inducing short-day conditions (Carrera et al., 2000; Bou-torrent et al., 2011). As a result, auxin levels increase at the stolon tip, which is accompanied by the reduction of GA levels in the sub-apical part of the stolon (Roumeliotis et al., 2012a; 2012b). This ceases elongation of the stolon and initiates swelling at the sub-apical region, followed by accumulation of starch and storage proteins to favour tuber maturation. Hence, the spatio-temporal changes in expression profiles of auxin and GA metabolism genes are crucial for early stages of stolon-to-tuber transitions. The changes in GA levels are controlled by regulation of *StGA20ox1* and *StGA2ox1* through StBEL5 (BEL1-like) and POTH1 (KNOX) homeodomain TFs as well as StSP6A protein. These factors act as positive regulators of tuberization (Banerjee et al., 2006a; Navarro et al., 2011; Mahajan et al., 2012). Specifically, StBEL5-POTH1 heterodimer binds to the promoter sequences of *StGA20ox1*, and reduces its expression (Chen et al., 2004). This interaction occurs through a tandem TGAC core motifs present in the promoter sequences of the target genes. Overexpression of StBEL5 and StSP6A independently, resulted in an increased levels of *StGA2ox1* in stolons. Promoter of *StGA2ox1* showed the presence of two tandem TGAC motifs and StBEL5-POTH1 has been demonstrated to bind to this sequence and could induce gene expression (Lin et al., 2013; Sharma et al., 2016).

Zhang et al. (2020) have identified reproductive phasiRNAs that are essential to silence genes associated with early events of meiosis in rice. Previously, we identified several phasiRNAs that are potentially involved in regulating key tuberization genes in potato (Kondhare et al., 2018; 2021). Specific phasiRNAs were predicted to cleave GA metabolism and auxin signaling genes. In the present study, a well conserved miR482 family member, St-miR482c was predicted to cleave the transcript of *disease resistance* gene (*StRGA4*) to generate phasiRNAs. Further, one of the phasiRNAs, referred as siRD29(-) could target the transcript

of GA biosynthesis gene (*StGA3ox3*). The function of this *StGA3ox3* isoform is unknown in potato. To investigate the existence and functionality of the *Stu-miR482c-StGA4-siRD29(-)-StGA3ox3* gene regulatory module, we asked following questions. (1) Does *siRD29(-)* generate from the *Stu-miR482c*-cleaved *StGA4* locus? (2) Is this siRNA generated during stolon-to-tuber transition stages? (3) Whether *siRD29(-)* targets *StGA3ox3* in a tissue-specific manner? (4) What is the role of *StGA3ox3* in potato development? (5) Does this small RNA-target gene pair (*siRD29(-)-StGA3ox3*) mediate the stolon-to-tuber transitions?

Here, we have validated the generation of *siRD29(-)* from its *PHAS* locus (*StGA4*) and confirmed *StGA3ox3* as its target. Further, we showed that the *Stu-miR482c-StGA4-siRD29(-)-StGA3ox3* gene regulatory module is functional during stolon-to-tuber transitions, and the *StGA3ox3* isoform is potentially involved in GA₁ and GA₃ biosynthesis. Overall, our study revealed a novel phasiRNA-mediated regulation of GA biosynthesis gene during tuber development in potato.

3.2 Materials and methods

3.2.1 Plant materials and growth conditions

In this study, the photoperiod-responsive potato cultivar - *Solanum tuberosum* ssp. *andigena* 7540 was used. It tuberizes under short-day conditions (SD; 8 h light / 16 h dark). Wild-type (WT) *andigena* plants were propagated by sub-culturing stem nodal explants on Murashige and Skoog's (MS) media supplemented with 2% sucrose (w/v) (Murashige and Skoog, 1962). *In vitro* potato plants were grown in a plant growth incubator (Percival Scientific) at 24 °C under long-day (LD; 16 h light / 8 h dark) conditions. Five to six weeks old plants of *Nicotiana benthamiana* were used for *Agrobacterium*-mediated transient co-infiltration assays.

3.2.2 Gene expression analysis

For studying transcript accumulation, four tissue types (shoot-apex, leaf, node and stem) were harvested from two-week old soil-grown *andigena* plants. To investigate the effect of SD photoperiod, plants were grown in soil under LD conditions for 10 weeks until they attained 10-12 leaves. Subsequently, half of the plants were transferred to the tuber-inducing SD conditions, whereas the remaining plants were maintained under LD photoperiod. Tissue types (shoot-apex, leaf, node, and stem) along with samples comprising stolon-to-tuber transition stages (stolon, swollen stolon, and mini-tuber) were harvested at 10 and 14 days after their transfer to SD. Leaves were also harvested on the earlier time-points (4th and 7th day after

SD induction). All samples were collected between Zeitgeber time (ZT) = 2 and ZT = 4. Total RNA was isolated from 100 mg of crushed tissues using RNAiso Plus (DSS TAKARA) as per the protocol. Complementary DNA (cDNA) synthesis was carried out using 2 µg of total RNA, Superscript IV Reverse Transcriptase (SSIV-RT Invitrogen), and Oligo (dT) primers. For gene expression analysis, quantitative PCR reactions were performed on a CFX96 Real-Time System (Bio-Rad) using gene-specific primers (Supplemental Table S1). Reactions were prepared with SYBR green master mix (TAKARA Clontech), and incubated at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, gene-specific annealing temperature for 15 s, and extension at 72 °C for 15 s. Similarly, 2 µg of total RNA was reverse transcribed using stem-loop primers, to study sRNA expression as per the protocol by Varkonyi-Gasic et al. (2007). Quantitative PCR was performed with sRNA-specific forward and universal reverse primers (Supplemental Table S1). PCR specificity was checked by melting curve analysis. Data were analysed using $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3.2.3 Cleavage site mapping by 5' RLM-RACE assay

To validate targets of small RNAs, a modified 5' RNA Ligase-Mediated Rapid Amplification of cDNA Ends (5' RLM-RACE) assay was performed using the First Choice RLM-RACE kit (Ambion). Briefly, total RNA was extracted from stolons of WT potato (*andigena*) plants by TRIzol reagent and ligated to the 5' RNA adapter. cDNA synthesis was performed using gene-specific reverse primers (Supplemental Table S1). Two sets of PCRs were performed with adapter-specific forward primers and gene-specific reverse primers (Supplemental Table S1). PCR amplicons from secondary-nested PCRs were cloned in a sub-cloning vector pGEM-T-easy, and sequence verified to identify the cleavage sites of sRNAs on their target gene transcripts.

3.2.4 Phylogenetic analysis

The amino acid sequences of putative StGA3ox isoforms were retrieved from Arabidopsis, potato, tobacco and tomato genomes. The phylogenetic tree was constructed using T-COFFEE (<http://www.ch.embnet.org/soaware/TCoffee.html>) and represented with TreeDyn (v198.3) (Dereeper et al., 2008).

3.2.5 Gene constructs designs and generation of potato transgenic lines

To generate constitutive overexpression (OE) gene constructs, the full-length coding sequences of *StGA3ox3* (1313 bp) and *StRGA4* (3239 bp) were amplified by reverse

transcription PCR from the leaves of soil-grown andigena plants using primers listed in Supplemental Table S1. These sequences were then individually cloned into the binary vectors, such as pBI121 and pCAMBIA1300, downstream to a constitutive CaMV35S promoter, respectively. Henceforth, these constructs are referred as 35S::*StGA3ox3*-pBI121 and 35S::*StRGA4*-pCAMBIA1300. Similarly, *Stu*-miR482c precursor OE construct was generated in a binary vector pBI121 (referred as 35S::pre-miR482c-pBI121). For the antisense construct, a non-conserved 446 bp sequence of *StGA3ox3* (comprising 41 bp from 5' untranslated region [UTR] region and 401 bp upstream to 5' UTR) was cloned in an antisense direction downstream to the CaMV35S promoter into a binary vector pBI121. To analyse *StGA3ox3* promoter activity, ~2.3 kb sequence upstream of *StGA3ox3* gene (including 5' UTR of 41bp) was amplified from genomic DNA of andigena plants and cloned upstream to the *GUS* reporter gene (*uidA*) in the pBI101 to generate *promStGA3ox3::GUS*-pBI101 construct. All the gene constructs were then transformed into *Agrobacterium tumefaciens* strain GV2260. Transgenic potato plants were generated using *Agrobacterium*-mediated leaf transformation method (Banerjee et al., 2006). Transgenic line of andigena harbouring 35S::*GUS* construct was considered as a vector control (VC). Phenotypic characteristics, such as plant height, internodal distance, leaf length and shape in transgenic lines were recorded before the initiation of SD photoperiod, whereas the tuber and root biomass were measured after four-weeks of SD induction. Moreover, *StBEL5* coding sequence (2064 bp) was PCR amplified from cDNA of *Solanum tuberosum* ssp. andigena (Supplemental Table S1) and into pBI221 vector to generate *StBEL5* OE construct.

3.2.6 *Agrobacterium* mediated co-infiltration assays

To understand *in planta* phasiRNA production, *Agrobacterium*-mediated co-infiltration assays were performed in leaves of 6-weeks old WT and RDR6 mutant plants of *N. benthamiana*. 35S::*StRGA4*-pCAMBIA1300 and 35S::pre-miR482c-pBI121 constructs were transformed into *Agrobacterium tumefaciens* GV3101 strain. For co-infiltration assays, individual *Agrobacterium* colony harbouring respective gene construct was inoculated in 3 ml LB broth containing appropriate antibiotics and grown overnight (primary culture). 100ml secondary cultures (having 150 μ M acetosyringone) were set up without any antibiotics. When the secondary culture reached an OD₆₀₀ of 1.00, cells were centrifuged and pellet was resuspended in a buffer (10 mM MgCl₂, 10 mM MES-K: pH= 5.6, plus 150 μ M acetosyringone), incubated at room temperature on a tube-roller for 3 hrs, followed by co-infiltration into the leaves of *N. benthamiana* plants using a needleless syringe. Co-infiltrations

with *agrobacteria* containing pBI121 and pCAMBIA1300 served as vector controls. Leaves were harvested post 48 hrs of co-infiltrations, and subjected further for sRNA analysis. For *in planta* validation of *StGA3ox3* as a target of siRD29(-), co-infiltrations were carried out in the leaves of WT *N. benthamiana* plants with 35S::*StRGA4*-pCAMBIA1300, 35S::pre-miR482c-pBI121, and 35S::*StGA3ox3*-pBI121 constructs.

Whether *StBEL5* regulates *StGA3ox3* gene expression, *Agrobacterium*-mediated co-infiltration assays were performed in leaves of *promStGA3ox3::GUS* lines (3-4 weeks old soil-grown plants). The 4th leaf of each plant was infiltrated with the 35S::*StBEL5* gene construct. Leaves were harvested 48 hrs post-infiltration and processed for *GUS* transcript accumulation and protein activity.

3.2.7 Dot blot analysis

Dot blot was carried out for sRNAs. Briefly, RNAs (2.5, 5 and 10 µg) were re-suspended in deionised formamide (Merck) heated at 98 °C for 2 min. Sample (2 µl) was loaded on the hybond N⁺ membrane (Ameshram) several times till the requisite amount of RNA is obtained on a single dot area. Further, the blot was UV cross-linked and hybridization was performed at 35 °C for 24 hrs in UltraHyb-oligo buffer (Ambion) with the oligo probes (Supplemental Table S1) end labelled with ³²P by poly-nucleotide kinase (Invitrogen). Blots were washed with 2X SSC buffer + 0.1% SDS thrice for 15 min at 35 °C. Blots were exposed to phosphor screen for 5 days and signal was captured using Typhoon phosphorimager (GE healthcare).

3.2.8 Analysis of *StGA3ox3* promoter activity

To analyse *StGA3ox3* promoter activity, four *promStGA3ox3-GUS* transgenic andigena lines were generated. All the lines showed a similar GUS activity pattern across the tissue types. Off these, one representative line was chosen for further analysis. Plants for *promStGA3ox3-GUS* transgenic line were grown *in-vitro* for five-weeks under LD conditions before transferring to the soil. They were grown under LD conditions until they attained 10-12 leaves. Half of the plants were subjected to SD induction for 14 days, while remaining half continued to grow under LD. Stolon-to-tuber transition stages as well as *in-vitro* grown plantlets (4-week old) were used to perform GUS assay as described in Jefferson (1987). Briefly, samples were kept for overnight in GUS extraction buffer at 37 °C and transferred to the series of ethanol gradients from 20 to 100% for the complete removal of chlorophyll. Samples were photographed in stereo microscope (Olympus MVX10). Fluorometric MUG

analysis from the subset of tissue samples (mentioned above) was performed as described (Jefferson et al., 1987). Frozen samples were homogenized in GUS extraction buffer (100 μ l). Samples were centrifuged at 16000 g for 5 min. Clear supernatant was used for protein estimation by Bradford's method (Bradford, 1976). Aliquots of extracts with approximately 10 μ g of protein were made and their volume was made up to 150 μ l with extraction buffer. GUS assay buffer (5 μ l) was added to each sample and incubated at 37 °C for 3 h. Reaction was stopped by adding stop (0.2 M Na₂CO₃) buffer. Fluorescence of the samples was measured using multimode plate reader (Ensign, Perkin-Elmer) with excitation wavelength 365 nm and emission wavelength 455 nm.

3.2.9 Histology and microscopy

To visualize the cell-type specific GUS expression, the tissues were fixed in 0.1 mM sodium phosphate buffer containing 4% paraformaldehyde (Sigma) and 2% glutaraldehyde overnight at 4 °C (Barker et al., 2021). The fixed tissues were then dehydrated in a series of ethanol gradient from 20 to 100% (v/v), followed by washes with xylene (JT Baker). Tissue blocks were prepared with molten paraplast (Leica). Ten micrometer sections were cut with the help of Microtome (Leica) and mounted with DPX on glass slides. Peels from abaxial side of GUS stained leaves were mounted with 10% glycerin solution to visualize guard cells.

3.2.10 Hormone treatment

Fifth leaf of five week old plants were treated with or without 100 μ M Jasmonic acid, 50 μ M GA₃, 50 μ M 2, 4-D and 0.25mM SA in different sets. Plants were then incubated in growth chamber (16 h light, 22°C/ 8h dark, 20°C) for 48hrs before collecting leaf samples.

3.2.11 Estimation of chlorophyll and anthocyanin content

Terminal leaflet of the 5th leaf (from the shoot apex) was excised from plants post four weeks of soil transfer for the purpose of chlorophyll and anthocyanin estimation. Using 100 mg of freeze-dried leaf tissues, chlorophyll was extracted in 80% acetone and the absorbance of supernatant was measured at 645 nm and 663 nm wavelengths, and total chlorophyll content (mg.g⁻¹) was calculated per the report of Bhattacharjee and Sharma (2012). Anthocyanin estimation was performed with a protocol described by Nakata and Ohme-Takagi (2014). Five volumes of extraction buffer (45% methanol and 5% acetic acid) were mixed with 100 mg of plant tissues and mixed thoroughly. Samples were centrifuged at 12000 g for 10 min at room

temperature and the absorbance (Abs) of the supernatant was measured at 530 nm and 657 nm, and the anthocyanin content, expressed as Abs 530 g FW⁻¹.

3.2.12 *In vitro* tuberization

A modified version of the method described by Hendriks et al. (1991) was used for *in vitro* tuberization assays. Single node cuttings of WT andigena, VC, and three types of transgenic potato lines (*StGA3ox3-OE*, *StGA3ox3-AS* and *StRGA4-OE*) were grown *in vitro* for 4-weeks under LD conditions on MS media containing 2% sucrose and 0.2% phytigel (Sigma-aldrich). Four consecutive nodes from the apex (excluding apical region) containing a dormant single axillary bud from 4-week old *in-vitro* grown plants were transferred to the tuber inducing MS media with 8% sucrose, 0.2% phytigel and incubated in the dark at 18 °C for 2 weeks and number of tubers formed were scored on alternate days. For each independent line, more than 80 single nodes (n>80) were used. Moreover, to study the effect of GA inhibitor treatment, 48 single nodes of transgenic lines, WT and VC plants were incubated on tuber inducing medium supplemented with GA biosynthesis inhibitor - paclobutrazol (0.5 mg/L) or mock (control), and scored for the rate of tuber formation.

3.2.13 Small RNA sequencing

To identify the phase of siRD29(-) generation, 5th leaf of *N. benthamiana* WT plants were co-infiltrated with *Agrobacterium* containing either 35S::*StRGA4-pCAMBIA1300* and 35S::*pre-miR482c-pBI121* or only 35S::*StRGA4-pCAMBIA1300*. Whereas, the leaves co-infiltrated with pBI121 and pCAMBIA1300 served as vector controls. Total RNA was isolated from these samples as described before. For small RNA-sequencing, all three co-infiltrated samples (35S::*StRGA4-pCAMBIA1300* + 35S::*pre-miR482c-pBI121*, 35S::*StRGA4-pCAMBIA1300*, and pBI121 + pCAMBIA1300) were processed in duplicates. RNA integrity was checked on Agilent Bioanalyzer chip (Tapestation 2200). Six independent small RNA libraries were prepared and subjected to the deep sequencing.

3.2.14 Processing of sRNA reads for the identification of phasiRNAs

Sequencing reads of 51 bp in length were generated with the Illumina HiSeq-X sequencing platform (Bionivid Technology Pvt. Ltd.). Quality of raw reads was examined by NGSQCtoolkit. Raw reads were filtered for high-quality with Q-Score ≥ 20 . rRNA and tRNA reads are filtered out by sRNA workbench, and adaptor null reads were discarded. Redundant reads from co-infiltrated samples were used to identify *PHAS* locus with the help of

sRNAworkbench (version 4.7), keeping a cut-off of at least 2 abundances for each aligned read with a length preference to 21 and a p-value cut-off of 0.05.

3.2.15 Quantification of GAs from *Agrobacterium*-infiltrated potato leaves

For the quantification of GA levels (GA₁, GA₃ and GA₄), a modified version of the protocol (Shaikh et al., 2022) was followed. To test if StGA3ox3 is involved in the production of GA₁, GA₃ or GA₄, *Agrobacterium*-mediated co-infiltration assays were performed in leaves of 4-week old soil-grown WT plants. The 4th leaf was agro-infiltrated with the 35S::StGA3ox3 construct, and samples were collected 48 hrs after infiltration. Liquid N₂ pulverized tissue (100 mg) was extracted in 250 µL of 80% methanol containing 400 ng/ml of Adonitol as an internal standard to monitor response of the machine throughout the run. Samples were vortexed vigorously and centrifuged twice at 14000 rpm for 30 min at 4 °C. Extracts were ran using Phenomenex Gemini® C18 column (50 *4.6 mm, 5 µm, 110 Å) in AB Sciex X500R Ultra-Performance Liquid Chromatograph-Electrospray Ionization-Quadrupole Time of Flight (UPLC-ESI-QTOF) Mass Spectrometer, in a negative ionization mode with a flow rate of 0.5 ml min⁻¹ and a gradient solvent program of 16 min (0.0 min, 10% methanol/water; 3.0 min, 10% methanol/water; 6.0 min, 50% methanol/water; 7.0 min, 70% methanol/water; 10.0-12.0 min, 95% methanol/water; 13.0-16.0 min, 5% methanol/water). Formic acid (0.1 %; LC-MS grade) was added to both the solvents. For MS/MS, the following parameters, ion spray voltage (V): -4500, de-clustering potential (V): -50 V and collision energy: -35V were used.

3.2.16 Gibberellin (GAs) quantification from transgenic lines

Shoot-apices as well as stolons of transgenic lines (*StGA3ox3-OE*, *StGA3ox3-AS* and *StRGA4-OE*) and WT plants grown in soil for six weeks under long day conditions were used for GA estimation. GAs were extracted according to published protocol (Simura et al., 2018; Jathar et al., 2022; Kundu et al., 2022) at NIPGR Metabolomics Facility, New Delhi. Lyophilized plant sample (35 mg) was extracted with 1 mL of cold extraction buffer (MeOH: H₂O: HCOOH, 15:4:0.1) consisting with 20 ng of [²H₂] GA₄ as an internal standard. Homogenized sample was centrifuged at 12,000 g for 10 min at 4 °C. Supernatant was taken out and loaded onto a Strata-X (Phenomenex ®) C₁₈ solid phase extraction (SPE) column pre-conditioned with 1 mL of methanol and 1 mL of 0.1% formic acid in water. After loading, the SPE column was washed twice with 0.1% formic acid and 5% methanol. The sample was eluted with 1 mL 0.1% formic acid in acetonitrile and dried in speed-vac. Dried sample was re-suspended in 50 µL 5% methanol and analysed by liquid chromatography coupled with a

SCIEX 6500⁺ triple-quadrupole-trap MS/MS. LC-MS/MS was performed. Separation of GAs (GA₁, GA₃, GA₄, GA₇, GA₈, GA₁₂ and GA₂₀) was carried out with a Zorbax Eclipse XDB C₁₈ column (50 × 4.6 mm, 1.8 µm, Agilent Technologies). The mobile phase comprised solvent A (water, 0.05% formic acid) and solvent B (acetonitrile) with the following elution profile: 0-0.5 min, 5% B; 0.5-5 min, 95% B; 5.5-5.6 min 95-5% B; and 5.6-7.5 min 5% B, with a flow rate of 0.5 mL min⁻¹. The column temperature was maintained at 27°C. For detection, the mass spectrometer was operated in negative ionization mode (MRM modus) to monitor analyte parent ion → product ion transition (345→143 for GA₃, 331→ 213 for GA₄, 329→ 211 for GA₇, 363→ 257 for GA₈, 331→ 313 for GA₁₂, 331.1→ 243 for GA₂₀ and 333.4→ 259.2 for [²H₂] GA₄). Mass spectrometer settings were as follows: ion spray voltage, -4500 eV; turbo gas temperature, 500°C; nebulizing gas, 50 psi; curtain gas, 45 psi and target scan time of 280msec. Analyst 1.7 software (Applied Biosystems) was used for data acquisition and processing.

3.2.17 Grafting

StGA3ox3-OE (# OE3), *StGA3ox3-AS* (# AS1) lines and WT plants were maintained *in vitro* on MS medium for three weeks before transferring to the soil. Lines were grown in soil for 4 weeks (16 h light, 22°C/ 8h dark, 20°C). Thereafter, three combinations of homo-grafts (WT/WT; OE/OE and AS/AS) and four types of hetero-grafts (WT/OE; OE/WT; WT/AS and AS/WT) were prepared as per the protocol described earlier (Bhogale et al., 2014). Grafts were incubated for 15 days in LD for recovery before being transferred to SD conditions for four week and scored for tuberization phenotype.

3.2.18 Accession numbers

Table 3.1 Following are the accession numbers of the genes under study.

Sr. No.	Gene Name	Accession	Source
1	<i>StGA3ox3</i>	Soltu.DM.01G018850.1	PGSC
2	<i>StRGA4</i>	Soltu.DM.09G019040.1	PGSC
3	<i>NbRDR6</i>	NM_001325654.1	NCBI
4	<i>NbL23</i>	XM_016629168.1	NCBI
5	<i>StBEL5</i>	PGSC0003DMT400076435	PGSC
6	<i>StGA2ox1</i>	PGSC0003DMT400054348	PGSC
7	<i>StGA3ox2</i>	PGSC0003DMT400054354	PGSC
8	<i>StGA20ox2</i>	PGSC0003DMT400071866	PGSC

9	<i>eIF3e</i>	PGSC0003DMT400076704	PGSC
10	Stu-miR482c	MI0020244	miRBase
11	Nb-miR168	MI0021352	miRBase
*PGSC- Potato Genome Sequencing Consortium (http://spudb.uga.edu/)			
* TAIR – The Arabidopsis Information Resource: (https://www.arabidopsis.org/)			
*miRBase- the microRNA database (https://www.mirbase.org/)			

3.2.19 Primer Sequences

Primers used in this chapter are listed in Table 3.2

Sr. No.	Primer name	Sequence (5'→3')
For Cloning purpose		
1	StGA3ox3-CDS-BamHI-FP	GAGAGGATCCGTTTTTTTTGTCAAAAGTCCA
2	StGA3ox3-CDS-SacI-RP	GAGAGAGCTCCTACAAATAATTTTCATGGTATATATG
3	StGA3ox3-AS-SacI-FP	GAGAGAGCTCCTGACTCTTTTGAGGGAC
4	StGA3ox3-AS-SpeI-RP	GAGAGGATCCAGTAGAAGTAGCCATTATTTTG
5	PromStGA3ox3-BamHI -FP	GAGAGGATCCGAAGGACACCCTCACATGC
6	PromStGA3ox3-BamHI -RP	GAGAGGATCCTATTTTGGACTTTTGACAAAAAAACAA
7	StRGA4-CDS-SalI-FP	GAGAGTCGACCAAAACATGGCAGACGCTTTC
8	StRGA4-CDS-KpnI-RP	GAGAGGTACCGGGCAATCCATTTTACTCAGC
9	premiR482c-BamHI-FP	GAGAGGATCCGAGAGATTTGGGAAGCTTTTGG
10	premiR482c-SacI-RP	GAGAGAGCTCGAGTAAATGATTGGAAGCCATTG
11	StBEL5-BamHI-FP	GGAGGATCCATGTACTATCAAGGAACCTCGG
12	StBEL5-SacI-cMYC-RP	AAAGAGCTCTCACAGATCTTCTTCAGAGATCAGTTTCTGTTC TGTTCTAGATTACCTGTAA
For transgenic line confirmation purpose		
13	NOST Scr-RP	GCAACAGGATTCAATCTTAAG
14	HYG-FP	CGGGGATTCCCAATACGAGG
15	HYG-RP	ATTTGTGTACGCCCCGACAGT
16	CamV35S-FP	CAACAAAGGGTAATATCCGGAAACC
17	CamV35S-RP	GAACTTCCTTATATAGAGGAAGGG
18	Kan-qFP	GACCACCAAGCGAAACATCG
19	Kan-qRP	AAAAGCGGCCATTTTCCACC
For 5'RLM-RACE and qPCR purpose		
20	StRGA4-482csite-FP	CTATGGAAGGGACAAAGAGCAGG
21	StRGA4-482csite-Rpi	GGGATTGAAATGCTCAGTCACTCTC
22	StRGA4-482csite-RPo	GAAGCCAAGTCTCAACATCCAG
23	StGA3ox3-siRD29-FP	AGAAACTTTGGCCACATGACTATC
24	StGA3ox3-siRD29-Rpi	AATTGTAGGGCTCCTGCAAATTCA
25	StGA3ox3-siRD29-RPo	TGAATCTGTGTGAGGAGCTAACCC
For real time PCR analysis purpose		
26	StGA3ox3-qFP	AAGAGTATCTTGGTCTCAAGGCT
27	StGA3ox3-qRP	GGATGGTGAGCAGATTACAAGATG

28	GUS-qFP2	GGCGATTAAAGAGCTGATAG
29	GUS-qRP2	ATCGGTGTGAGCGTCGCAGA
30	StGA3ox2_34470FP	TCGATAGAATTGGCCATGCATGC
31	StGA3ox2_34470RP	CGAGCAACCCCATAGCCAG
32	StGA2ox1_4348FP	AACGGAAGGTTTATGAGTGTGAGG
33	StGA2ox1_4348Rpi	TGCTCATTAGGTTCCCTCCATTAGAC
34	EIF3e qFP	GGAGCACAGGAGAAGATGAAGGAG
35	EIF3e qRP	CGTTGGTGAATGCGGCAGTAGG
36	StGA20ox2_71866FP	CTACAAGGCTCGTCCCACAT
37	StGA20ox2_71866RP	TCGCATTGCAGTATTCTTGG
38	StBEL5 qFP	AGGGATACTCTTATATTGTGTGAG
39	StBEL5 qRP	GTCTCCACTTCTTTTCTCCTATG
40	StlT_qF	AAGAGCTCGTCGCCGTTAAA
41	StlT_qR	GCAGGTCTACGAGCTGTGTT
42	SP6A_qF	GACGATCTTCGCAACTTTTACA
43	SP6A_qR	CCTCAAGTTAGGGTCGCTTG
44	Nb_L23-FP	AAGGATGCCGTGAAGAAGATGT
45	Nb_L23-RP	GCATCGTAGTCAGGAGTCAACC
46	Nb_RDR6-FP	CTCAGCTTGGGGACCTCA
47	Nb_RDR6-RP	CAGCCTCCAGAATCCTCAC
For small RNA qPCR purpose		
48	siRD29(-)-STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATAC GAC CCTCAA
49	siRD29(-)-FP	CGGCGGG AATAGAACTTTCATC
50	StU6-STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATAC GACTTGGAC
51	StU6-FP	GACACGCACAAATCGAGAAATG
52	Stu-miR482C-STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATAC GACTTGGCA
53	Stu-miR482C-FP	CGGCGTTTCCTATTCCACCCA
54	NtmiR168-STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATAC GACGTCCCCG
55	NtmiR168-FP	TCGCTTGGTGCAGGTCCG
56	NtU6-STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATAC GACGGCCAT
57	NtU6-FP	GCGGGAACGATACAGAGAAGATTAGC
58	Univ-RP	AGTGCAGGGTCCGAGGT
For dot blot analysis		
59	siRD29(-)_RC	CCTCAAGATGAAAGTTCTATT

3.3 Results

3.3.1 MicroRNA482c cleaves *StRGA4*, a *PHAS* locus encoding *NB-ARC domain-containing disease resistance protein* in potato

Previously, we identified 830 putative phasiRNA producing (*PHAS*) loci from sRNA-sequencing data-sets of stolon-to-tuber transition stages in potato (Kondhare et al., 2018). Amongst them, we found that a *PHAS* locus (Chr09:46314301..46314552) can generate a phased siRNA (phasiRNA), which targets a gibberellin biosynthesis gene- *StGA3ox* (Soltu.DM.01G018850.1). This *PHAS* locus was located in the exonic region of a *NB-ARC domain-containing disease resistance protein* - *StRGA4* (Soltu.DM.09G019040.1) and predicted to be cleaved by Stu-miR482c to generate phasiRNAs (Table 3.4, Fig. 3.1 A). Through a modified 5' RLM-RACE, we confirmed that Stu-miR482c cleaves *StRGA4* transcript with a high frequency (8 of 8) at the predicted site (Fig. 3.1 B). Based on the cleavage site, the phase of phasiRNA that targets *StGA3ox* was mapped on *StRGA4* transcript at the 29th position on the antisense strand, hence referred as siRD29(-) (Fig. 3.1 A).

3.3.2 SiRD29(-) generation is RDR6-dependent

To test if microRNA482c (Stu-miR482c) is responsible for generating siRD29(-) from *StRGA4* transcript, we co-infiltrated Stu-miR482c and *StRGA4* OE constructs in leaves of *Nicotiana benthamiana* plants. Dot blot analysis indicated the accumulation of siRD29(-) in the leaves post 48 hrs of co-infiltration (Fig. 3.1 C). No siRNAs were generated in the empty vector infiltrated leaf samples (control). Further, we sequence verified this siRNA (Fig. 3.1 D, E). RDR6 is an essential factor in conventional biogenesis of phasiRNAs (Yoshikawa et al., 2005). To investigate if siRD29(-) is generated in RDR6-dependent manner, co-infiltration experiment was also performed in *RDR6-RNAi* line of *N. benthamiana*. These plants have a significantly reduced *RDR6* transcript level and thus have low levels of phasiRNAs (Schwach et al., 2005). Our analysis indicated that the generation of siRD29(-) was reduced by 96% in the co-infiltrated leaves of *RDR6i* plants compared to WT (Fig. 3.1 F). These results confirmed that siRD29(-) is generated from *StRGA4* transcript in RDR6-dependent manner post cleavage by Stu-miR482c.

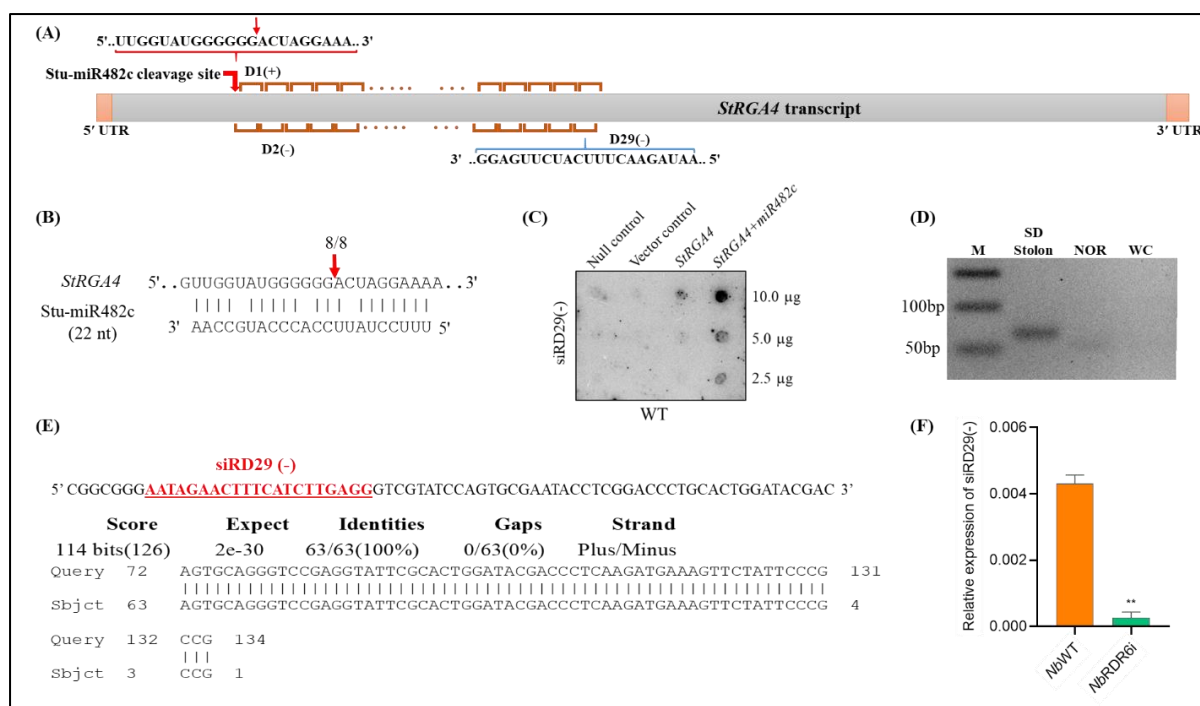


Fig 3.1 A phasiRNA siRD29(-) generates from *StRGA4* transcript. (A) A schematic representation of *StRGA4* transcript and Stu-miR482c cleavage site on *StRGA4* mRNA. Putative phasiRNAs and the location of a phasiRNA siRD29(-) on the antisense strand is also shown. The number in the bracket indicates the read count obtained in small RNA sequencing dataset from stolons (Kondhare et al., 2018) (B) A modified 5' RLM-RACE results depicting the cleavage site mapping of Stu-miR482c on *StRGA4* transcript. Arrow shows the site of cleavage. The numbers (8/8) represent the frequency of clones with the same cleavage site. (C) Dot blot based detection of a phasiRNA siRD29(-) in leaves of wild-type *N. benthamiana* (*NbWT*) plants following co-infiltration of gene constructs using *Agrobacterium tumefaciens*. The increased abundance of siRD29(-) was detected using the blot paper. Null control= mock infiltrated leaves; Vector Control= empty vector infiltrated leaves; *StRGA4*= leaves infiltration with 35S::*StRGA4* construct; and *StRGA4* + miR482c= leaves co-infiltrated with 35S::*StRGA4* and 35S::pre-miR482c gene constructs in a binary vectors. (D) Agarose gel image for siRD29(-) detection in SD-induced stolons using stem-loop RT PCR. M=50 bp ladder, NOR= stem-loop primer control, WC= water control. (E) Stem-loop PCR amplified product was cloned in to a sub-cloning vector pGEM-T-easy and sequence confirmed using multiple clones. The resulting representative sequence is shown. (F) Quantification of siRD29(-) levels from agroinfiltrated samples of WT plant and *RDR6i* line of *N. benthamiana* with 35S::*StRGA4* and 35S::pre-miR482c gene constructs. *NtU6* was used as reference gene.

3.3.3 A novel phasiRNA siRD29(-) targets *StGA3ox3*

The phylogenetic analysis revealed a close conservation of this isoform with *Arabidopsis AtGA3ox3* (NP_193900.1) (Fig. 3.2). Thus, we referred this gene as *StGA3ox3* henceforth.

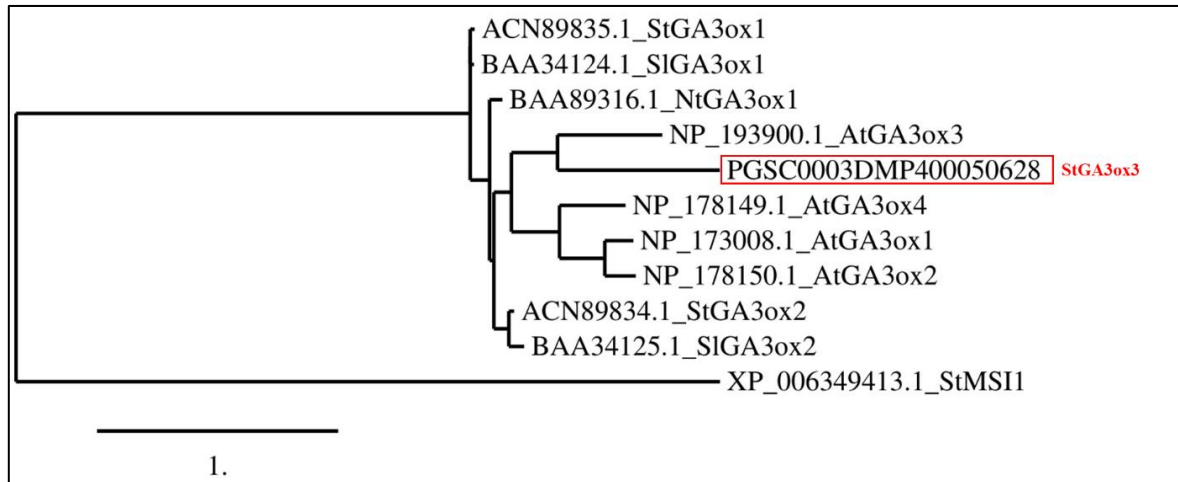


Fig 3.2 Phylogenetic relationship of GA3ox proteins. The deduced amino-acid sequences of GA3ox proteins from *Arabidopsis*, potato, tomato and tobacco were analyzed. StMSI1 (a potato non-GA3ox related protein) amino-acid sequence was included to root the tree. NCBI accessions for protein sequences used are written before the respective protein names in the phylogenetic tree. In the phylogenetic tree, the branch length is proportional to the number of substitutions per site and the tree is rerooted using midpoint rooting in TreeDyn. St, *Solanum tuberosum*; Sl, *Solanum lycopersicum*; Nt, *Nicotiana tabacum*; At, *Arabidopsis thaliana*. Based on the close conservation with *AtGA3ox3*, *StGA3ox* isoform (PGSC0003DMP400050628; Soltu.DM.01G018850.1) identified in this study in potato (highlighted in red box) is referred as *StGA3ox3* henceforth.

In silico analysis predicted that siRD29(-) targets *StGA3ox3* with an expectancy value of 2.5 ($E = 2.5$). Using a modified 5' RLM-RACE, we confirmed the cleavage of *StGA3ox3* transcript by siRD29(-) with a high frequency (9 of 9) at 11th/12th nucleotides position (Fig. 3.3 A). For *in-planta* validation of siRD29(-) targeting *StGA3ox3*, a transient co-expression of 35S::*StGA3ox3*, 35S::*miR482c* and 35S::*StGA4* was carried out in leaves of WT and RDR6i plants of *N. benthamiana*. *StGA4* and *Stu-miR482c* levels were significantly enhanced compared to mock and empty vector (control) infiltrated plants, and transcript levels of *StGA3ox3* in co-infiltrated samples were significantly reduced compared to control (Fig. 3.3 B). As expected, co-infiltrated RDR6i lines did not show any change in *StGA3ox3* levels due to low levels of siRD29(-) in these plants (Fig. 3.3 C). Altogether, these results suggest that siRD29(-) acts in a *trans*-manner to regulate *StGA3ox3*.

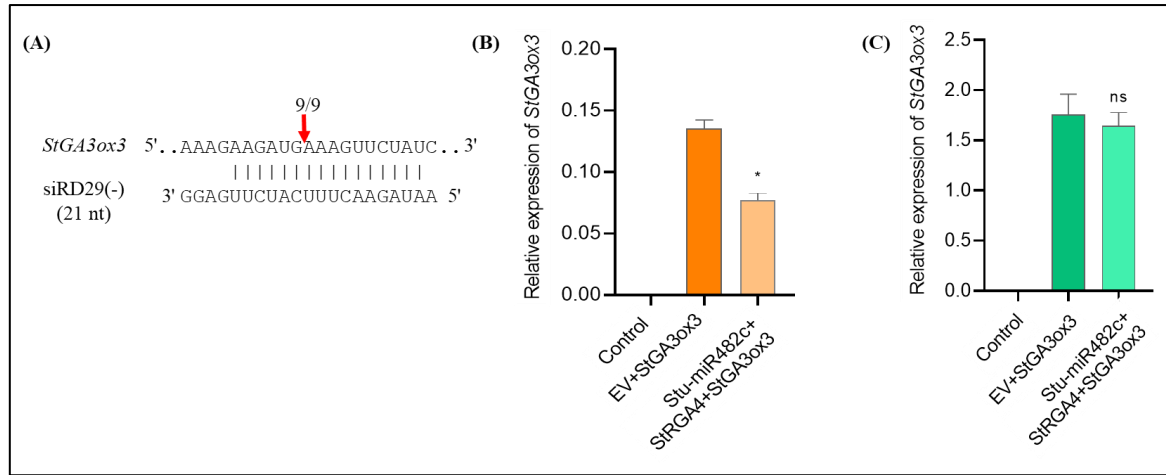


Fig. 3.3 siRD29(-) targets *StGA3ox3* mRNA post-transcriptionally. (A) A modified 5' RLM-RACE results representing the cleavage site mapping of a phasiRNA, siRD29(-) on *StGA3ox3* transcript. The numbers (9/9) represent the frequency of clones showing the same cleavage site. (B) *StGA3ox3* mRNA accumulation 48 h after co-infiltration in WT *N. benthamiana* leaves. Control= Mock infiltrated leaves; EV + *StGA3ox3* = leaves co-infiltrated with empty vector and 35S::*StGA3ox3* construct; Stu-miR482c + *StGA3ox3* = leaves co-infiltrated with 35S::pre-miR482c, 35S::*StGA3ox3* gene constructs. The transcript abundance was plotted as $2^{(-\Delta Ct)}$ method (Livak and Schmittgen, 2001). (C) *StGA3ox3* mRNA accumulation 48 h after co-infiltration in *RDR6i N. benthamiana* leaves. Error bars indicate standard error of means (SEM) from three biological replicates (n=3). * indicates $p < 0.05$ using one-way ANOVA. ns = not significant at $p < 0.05$.

Further, quantitative PCR analysis revealed that siRD29(-) was highly expressed in leaves, followed by shoot-apex and stolons under LD condition (Fig. 3.4 A). A significant increase was seen in siRD29(-) levels after 4 and 7 days of SD induction (Fig. 3.4 B). During stolon-to-tuber transition stages, it showed highest expression in SD-induced stolons (Fig. 3.4 C).

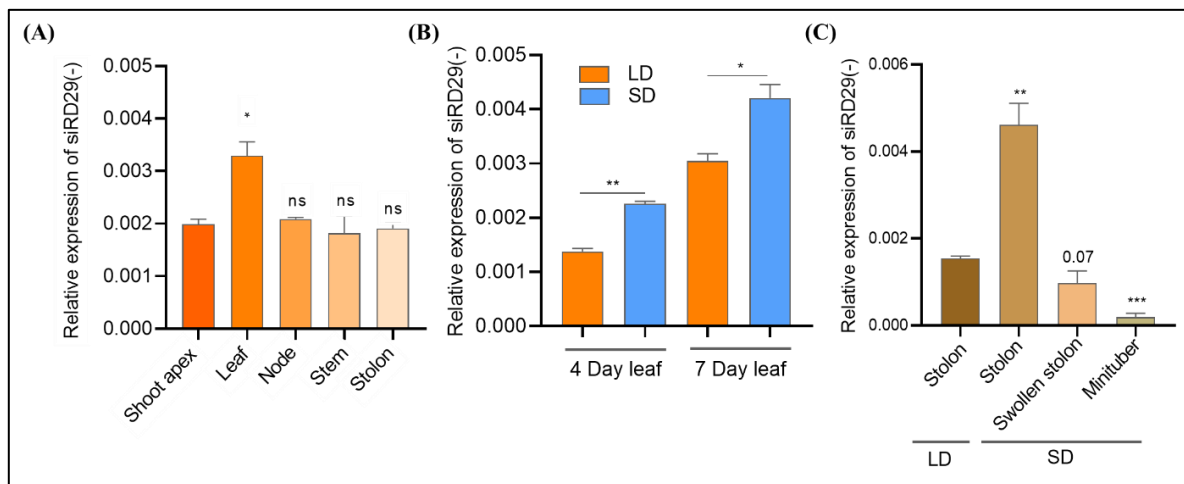


Fig. 3.4 Expression of siRD29(-) in potato tissue types. (A) Expression of siRD29(-) from the two-week old potato plant. *StU6* was used as reference gene. Different tissue types (shoot-apex, leaf, stem, node, stolon) were used for the analysis. The abundance of siRD29(-) was plotted using $2^{-(\Delta Ct)}$ method (Livak and Schmittgen, 2001). (B) Effect of short-day (SD) photoperiod (4 and 7 days) on the expression of siRD29(-) in leaves of potato plants. (I) The levels of siRD29(-) during stolon-to-tuber transition stages. Error bars indicate standard error of means (SEM) from three biological replicates (n=3). Asterisks (one, two and three) indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.005$ respectively, using one-way ANOVA. ns = not significant at $p < 0.05$.

3.3.4 Stu-miR482c triggers secondary siRNAs from *StRGA4* locus *in planta*

To confirm the generation of secondary siRNAs from *StRGA4* post Stu-miR482c cleavage *in planta*, we performed small RNA-sequencing of *N. benthamiana* leaves co-infiltrated with Stu-miR482c and *StRGA4* OE constructs. sRNAs were isolated from the infiltrated leaves (48 hrs post infiltration), and subjected for deep-sequencing. The size distribution profile of sRNAs indicated that *StRGA4* expression did not have any effect on sRNA population from *N. benthamiana*, but an increase in the proportion of 21 and 22 nt small RNAs was observed in co-infiltrated samples as compared to controls (Fig. 3.5 A). Further, we mapped sRNA reads to the *StRGA4* transcript. *StRGA4* specific sRNA reads were absent in vector control samples, but were abundant in co-infiltrated samples in presence of Stu-miR482c (Fig 3.5 B). The secondary siRNAs phase begun at the 1st nucleotide from the Stu-miR482c cleavage site, which extended up to 29th position, where siRD29(-) is located (Fig. 3.5 C). Subsequently, the number of reads for siRNAs reduced (Table 3.5). The first three phasiRNAs (D1-D3) from the *StRGA4* locus had more number of sRNA reads (Table 3.5). Though, the position of siRD29(-) was far from the Stu-miR482c cleavage site, we still obtained higher number of sRNA reads in comparison with other siRNAs near to the cleavage site (Fig. 3.5 C). Overall, high throughput sequencing of co-infiltrated samples confirmed that siRD29(-) is generated post Stu-miR482c cleavage at the 29th phase from the *StRGA4 PHAS* locus.

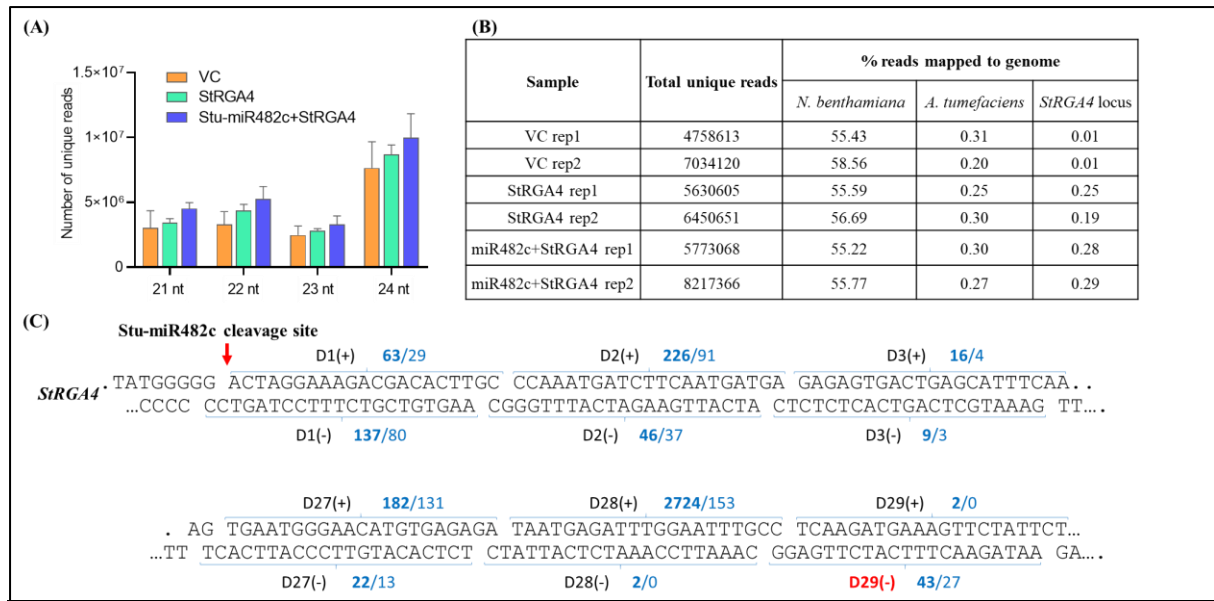


Fig. 3.5 A phasiRNA siRD29(-) is generated from the antisense strand of *StRGA4* transcript at the 29th phase post Stu-miR482c-mediated cleavage. (A) Abundance of small RNAs (21-24 nt) in co-infiltrated leaf samples of *N. benthamiana*. VC= leaves infiltrated with empty vector; *StRGA4*= leaves infiltrated with 35S::*StRGA4*; *StRGA4* + Stu-miR482c= leaves infiltrated with 35S::*StRGA4* and 35S::*pre-miR482c* constructs. (B) Total read counts from small RNA-sequencing of infiltrated samples of *N. benthamiana* for control, *StRGA4* and co-infiltration of 35S::*StRGA4* and 35S::*pre-miR482c* gene constructs. The mapping percentage of unique reads to the genome of *N. benthamiana*, *Agrobacterium tumefaciens* and *StRGA4* locus are also shown. (C) Phased siRNAs register with Stu-miR482c guided cleavage site (indicated by a red arrow). Phased siRNAs (21 nt) are marked with the position from the cleavage site. The numbers above and below indicated the reads obtained from the leaf samples co-infiltrated with *StRGA4* and pre-miR482c gene constructs versus those infiltrated with only *StRGA4* gene construct. For a simplified view, phasiRNAs generated from the first three phases and those from 27th to 29th positions are represented.

3.3.5 *StGA3ox3* is highly expressed in the swollen stolon under SD

StGA3ox3 showed a ubiquitous expression in all tissue types tested, with the highest expression in leaves, followed by node, shoot-apex and stem tissues (Fig. 3.6 A). The effect of SD photoperiod was seen to be gradual as the leaves from WT plants did not show a significant reduction in *StGA3ox3* levels after 4 days of SD induction, but it was reduced 7 days post SD induction (Fig. 3.6 B). Further, at 14 days of SD, *StGA3ox3* expression significantly reduced in leaves, petioles and nodes, but it was high in the shoot-apex compared to LD conditions (Fig. 3.6 C). The expression of *StGA3ox3* remains unchanged in root and stem tissues under LD/SD

conditions (Fig. 3.6 C). It was evident that *StGA3ox3* was highly expressed in swollen stolons, but its expression reduced in mini-tubers (Fig. 3.6 D).

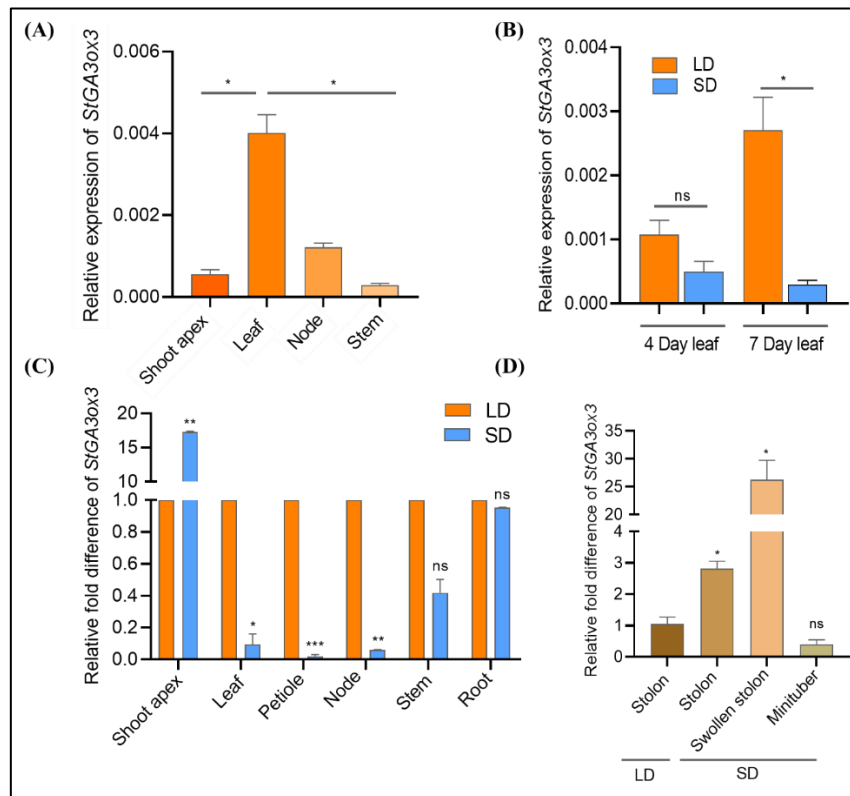


Fig. 3.6 Expression analysis of *StGA3ox3* in different tissues of potato cultivar *Andigena*. (A) Accumulation of *StGA3ox3* transcript in various tissues of WT andigena potato plants. Four tissue types of 2 week old soil-grown potato plants, such as shoot-apex, leaf, node and stem were used for analysis. *StEIF3e* was used as a reference gene. Statistical significance was calculated in comparison to the leaf transcript abundance. (B) Effect of short-day (SD) photoperiod (4 and 7 days) on the expression of *StGA3ox3* in leaves of potato plants. (C) Effect of photoperiod on the expression of *StGA3ox3* in different tissue types of potato plants, subjected to long-day (LD) or SD induction for 14 days, post 8 weeks in soil under LD conditions. Fold-change of the gene expression in each tissue type for SD treatment is compared to respective LD expression. (D) Gene expression of *StGA3ox3* during stolon-to-tuber transition stages (stolon, swollen stolon and mini-tuber) harvested from potato plants post 10 days of SD or LD induction. Fold-change of the gene expression in SD is compared to LD for each tissue type. Asterisks (one, two and three) indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.005$, respectively, using One way-ANOVA Student's t-test. ns = not significant at $p > 0.05$.

However, the expression of other known GA metabolism genes (*StGA3ox2*, *StGA20ox2* and *StGA2ox1*) and tuberization activator *StBEL5* during stolon-to-tuber transition stages were in line with the previous reports (Fig. 3.7).

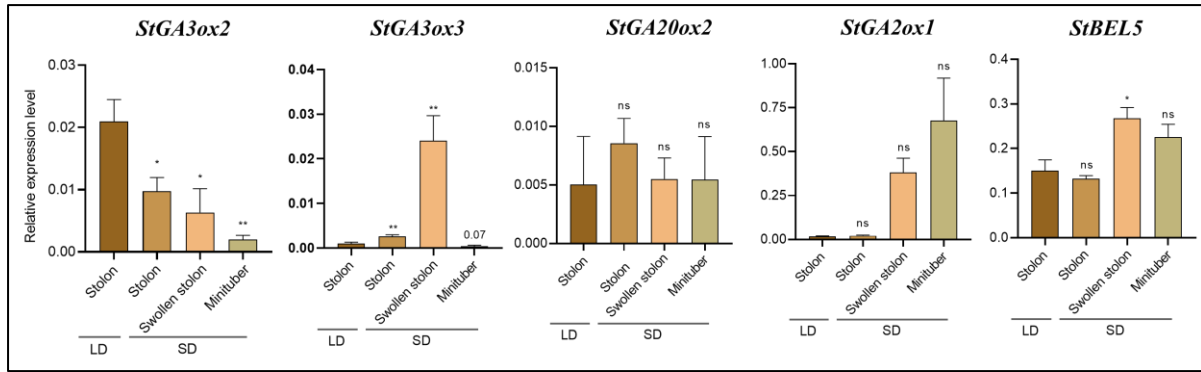


Fig. 3.7 Expression analysis of GA metabolism genes during stolon-to-tuber transitions under SD. Expression of GA biosynthesis genes, such as *StGA3ox2* (A), *StGA3ox3* (B), and *StGA20ox2* (C), GA catabolism gene, *StGA2ox1* (D) and *StBEL5* (E) in stolons, swollen stolons and mini-tubers under LD or SD conditions. *StEIF3e* was used for normalization of gene expression data. Error bars indicate standard error of means (SEM) from three biological replicates (n=3). Asterisks (one and two) indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively, using the one-way ANOVA. ns = not significant at $p > 0.05$.

The pattern of GUS activity using 5-week old *in-vitro* plants of *promStGA3ox3::GUS-pBI121* lines matched with *StGA3ox3* gene expression. (Fig. 3.8 A). GUS expression was observed in leaves, roots, and at the junction of stem-petiole and stem-roots (Fig. 3.8 B, E, F, H, and I). In the leaf, GUS expression was prominently observed in the guard and mesophyll cells (Fig. 3.8 C-D), respectively. Longitudinal (Fig. 3.8 F) and transverse (Fig. 3.8 G) sections of the stem-petiole junction revealed that *StGA3ox3* promoter expresses in the cortex cells and not in the meristem region. Transverse section of roots (Fig. 3.8 I) indicated GUS expression in the cortex region. A faint GUS expression was also seen in the phloem region (Fig. 3.8 I). Strong GUS activity was observed in swollen stolons (Fig. 3K), whereas a faint GUS expression was noted in mini-tubers and elongated stolons (Fig. 3J and L, respectively). Similarly, *GUS* and *StGA3ox3* transcript expression was increased in swollen stolons, and was reduced in tubers (Fig. 3.8 M-N). We noticed that GUS protein activity had not changed in swollen stolons, but reduced at mini-tuber stage (Fig. 3.8 O). Since, the total protein content increases during stolon-to-tuber stages (Fig. 3.8 P), the effect of induced GUS expression did not correlate with the GUS protein activity in swollen stolons.

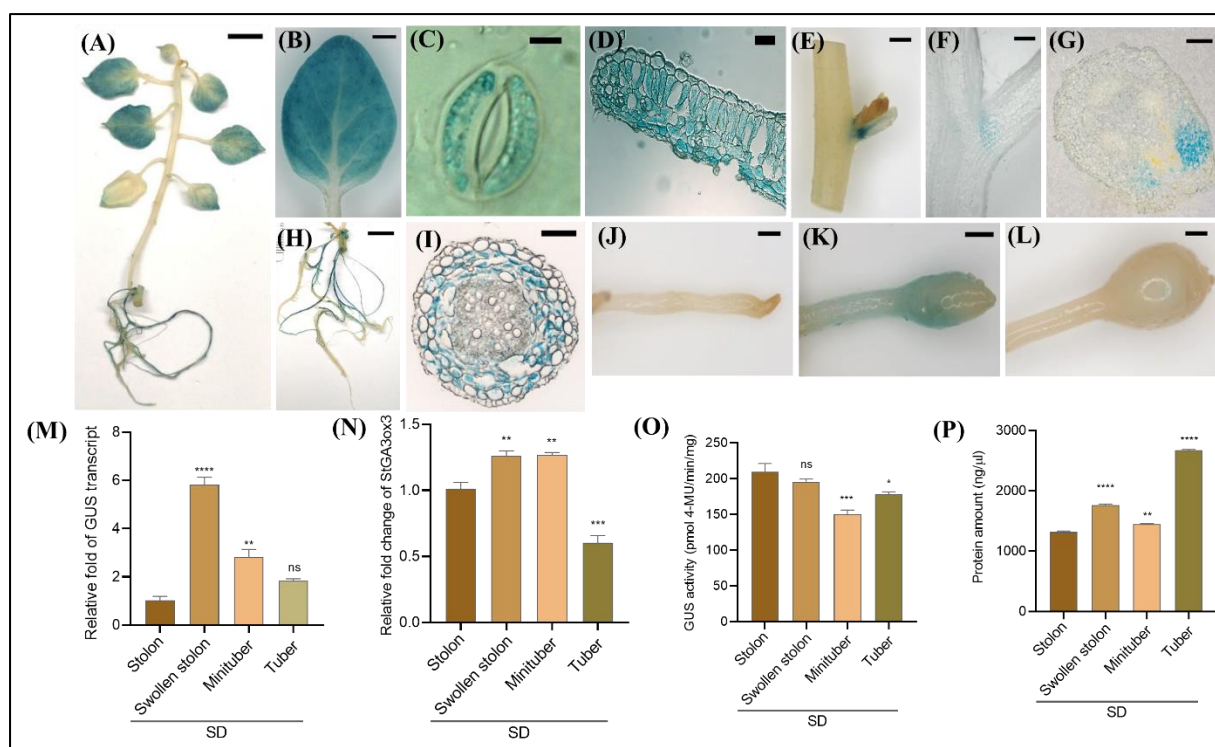


Fig. 3.8 *StGA3ox3* promoter exhibits tissue-specific GUS activity. (A) GUS activity in *promStGA3ox3::GUS* transgenic lines. GUS activity in 5-week old *in-vitro* grown entire plant, leaf and guard cells (B and C), longitudinal section of leaf blade (D), petiole-stem nodal junction (E), longitudinal and transverse sections of petiole-stem nodal junction (F and G), roots and their transverse sections showed GUS expression in the cortex region (H and I), stolons, swollen stolons and mini-tubers (J, K and L) harvested from 8 week old soil grown plants of *promStGA3ox3::GUS* induced with LD/SD conditions for 10 days. No visible GUS expression was observed in stolon (J). Arrows indicate GUS expression in specific cell-types. Scale bars, A, H= 1 cm; B, E, J, K and L= 1 mm; F= 500 μ m; G= 200 μ m; I= 100 μ m and C, D = 20 μ m. (M) The GUS transcript levels during stolon-to-tuber transition stages harvested from *promStGA3ox3::GUS* transgenic lines post 10 days of SD induction. (N) The expression analysis of *StGA3ox3* gene, (O) Quantification of GUS activity, and (P) The total protein content in stolons, swollen stolons and mini-tubers of *promStGA3ox3::GUS* transgenic lines under SD conditions. *StEIF3e* was used for normalization of gene expression data. Error bars indicate standard error of means (SEM) from three biological replicates (n=3). Asterisks (one, two, three and four) indicate significant differences at $p < 0.05$, $p < 0.01$, $p < 0.005$ and $p < 0.001$ respectively, using one-way ANOVA. ns = not significant at $p < 0.05$.

3.3.6 Altered expression of *StGA3ox3* affects leaf size and chlorophyll content with no effect on tuber weight

To characterize the function of *StGA3ox3* in potato, five constitutive *StGA3ox3-OE* lines in andigena were generated (Fig. 3.9 A). Of them, three independent lines (referred as

#OE2, #OE3, #OE5) with moderate or high levels of *StGA3ox3* expression were chosen for further analysis (Fig. 3.9 A-B).

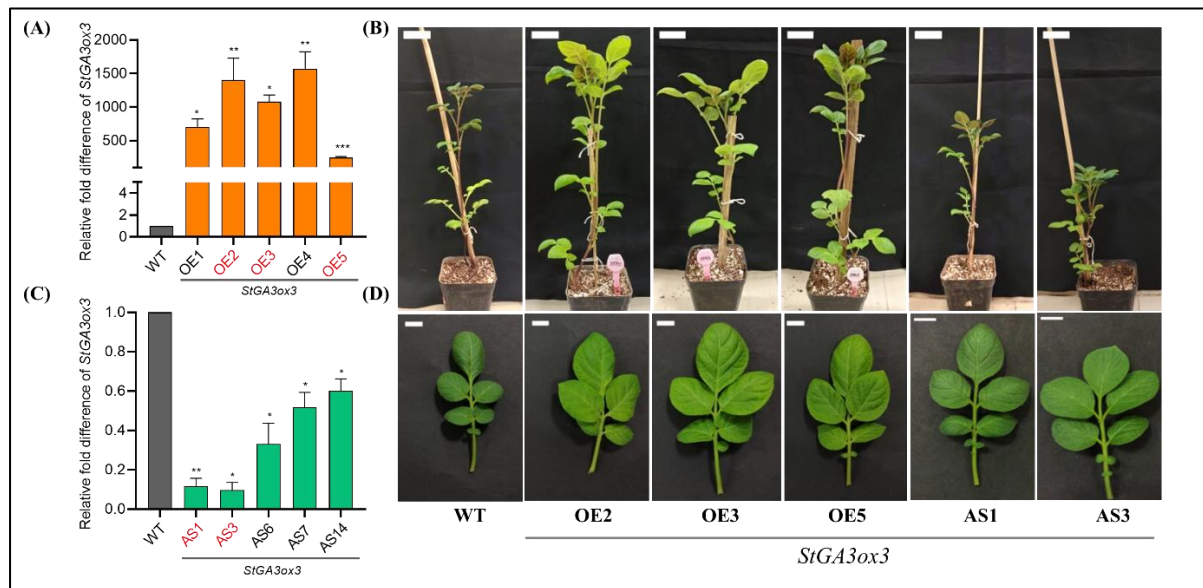


Fig 3.9 Overexpression (OE) and knockdown (AS) lines of *StGA3ox3*. A) Expression levels of *StGA3ox3* in the 5th leaf of overexpression and antisense lines compared to WT Lines underlined in red were taken for further experiments. *StEIF3e* was used as a reference gene for RT-qPCR analysis. Error bars indicate standard error of means (SEM) from three biological replicates (n=3). Asterisks (one, two and three) indicate significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively using Student's t-test. ns= not significant at $p > 0.05$. (B) Plant architecture of *StGA3ox3*-OE and -AS lines along with WT. Scale bar= 5 cm. (C) Representative images of the 5th leaf from WT, *StGA3ox3*-OE and *StGA3ox3*-AS lines. Scale bar= 1 cm.

OE lines showed an increase in plant height compared to WT (Fig. 3.10 A, C). Leaflet numbers were same but the overall leaf size was larger and the terminal leaflet length was significantly higher in OE lines than WT (Fig. 3.9 D; Fig. 3.10 B, D). However, no change in the total leaf length was noted (Fig. 3.10 F). Chlorophyll content of the leaves was reduced in *StGA3ox3*-OE lines (Fig. 3.11 A), whereas #OE3 had a reduced anthocyanin content compared to WT (Fig. 3.11 B).

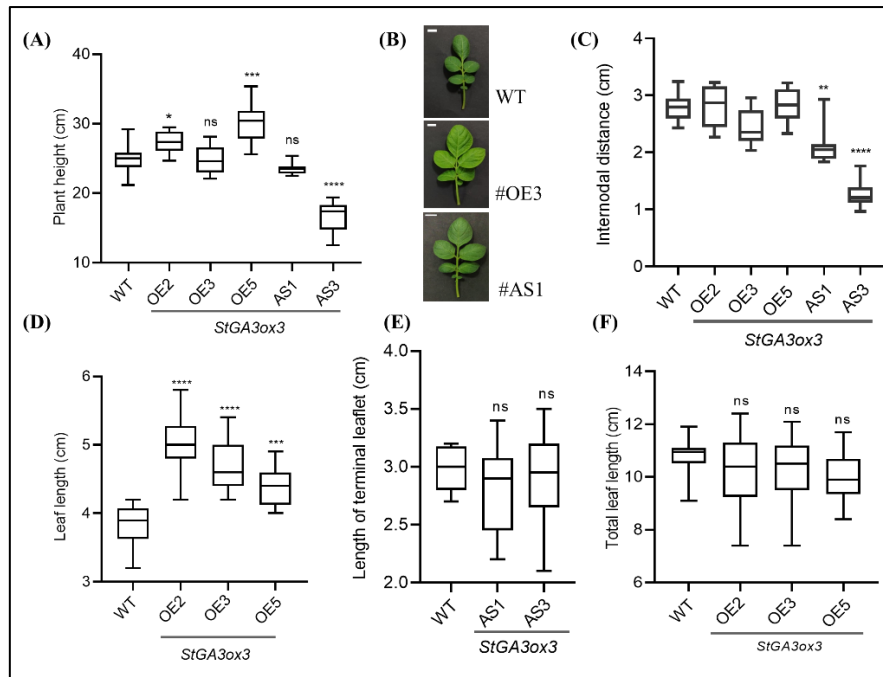


Fig. 3.10 Overexpression of *StGA3ox3* affects plant height and leaf size. (A) Plant height is represented in *StGA3ox3*-OE and -AS lines in comparison with WT (n=12). (B) Representative images of the 5th leaf from WT, *StGA3ox3*-OE and *StGA3ox3*-AS line. Scale bar= 1 cm (C) Plant inter-nodal distance in *StGA3ox3*-OE and -AS lines is shown in comparison to WT. (D) Terminal leaflet length of fully expanded 5th leaf has been shown in *StGA3ox3*-OE lines in comparison with WT. Data is from 12 independent plants for each line (n=12). (E) Terminal leaflet length from AS lines is represented (n=12). (F) Total leaf length for 5th leaf from shoot-apex in OE lines. Asterisks (one, two, three and four) indicate significant differences at $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively, using one way ANOVA. ns= not significant at <0.05 .

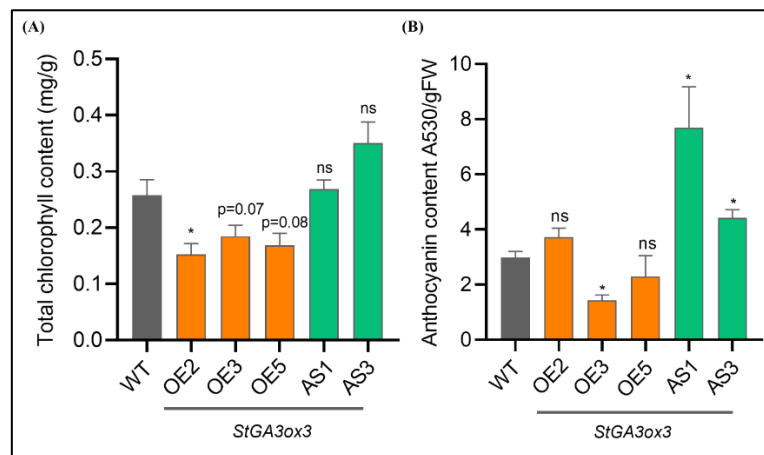


Fig. 3.11 *StGA3ox3* overexpression affects chlorophyll and anthocyanin content of leaves. (A) Total chlorophyll content from the 5th leaf of *StGA3ox3*-OE and *StGA3ox3*-AS lines. (B) Estimation of anthocyanin content in the 5th leaf from the shoot-apex in *StGA3ox3*-OE, *StGA3ox3*-AS lines and WT plants. * at $p < 0.05$, using one way ANOVA. ns= not significant at <0.05 .

To assess the effect of *StGA3ox3* knockdown, we generated its antisense (AS) lines in andigena (Fig. 3.9 C). We selected two antisense lines (referred as #AS1 and #AS3) with more than 80% reduction in the *StGA3ox3* transcript levels (Fig. 3.9 B-C). *StGA3ox3*-AS lines showed a reduction in plant height accompanied by a significant decrease in the inter-nodal distance (Fig. 3.10 A, C). Although there was no change in the length of terminal leaflet as observed in *StGA3ox3*-OE lines (Fig. 3.10 B, E), the leaflets of the *StGA3ox3*-AS lines were flattened compared to WT (Fig. 3.9 D; Fig. 3.10 B). Evaluation of the tuberization potential of OE and AS lines of *StGA3ox3* showed that the overall tuber weight remains unchanged in both transgenic lines (Fig 3.12 B-E). However, AS lines produced significantly higher tuber numbers per plant (Fig. 3.12 A, C).

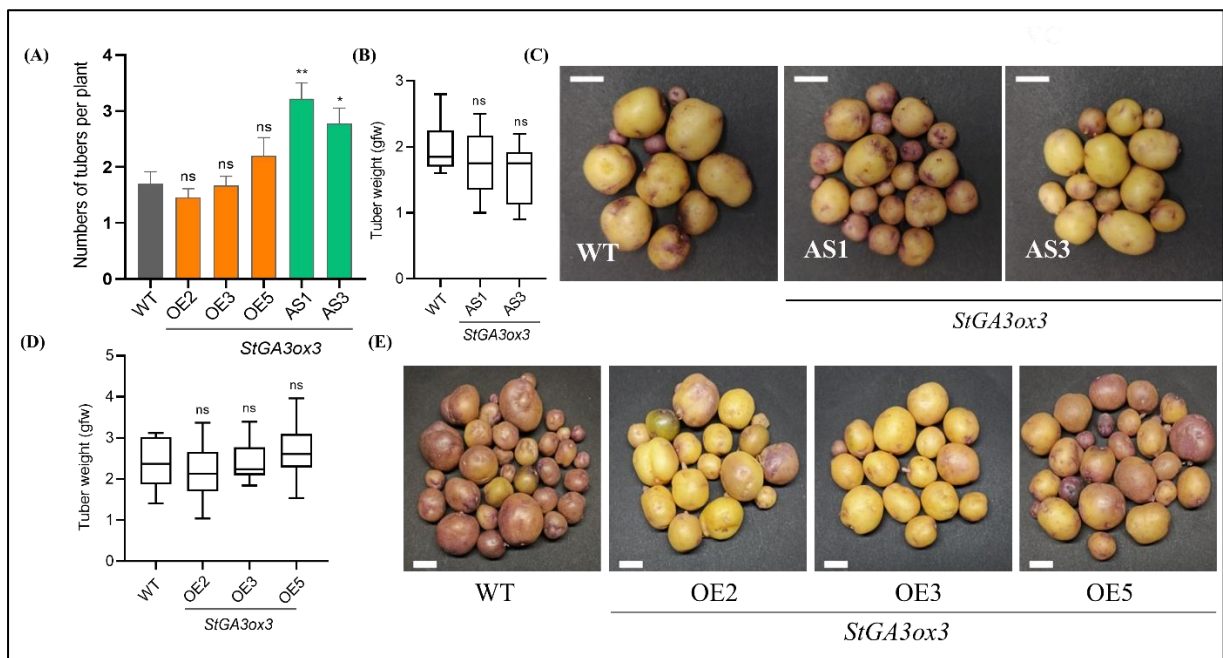


Fig. 3.12 Knockdown of *StGA3ox3* alters tuber numbers in potato. (A) Number of tubers in *StGA3ox3*-OE and *StGA3ox3*-AS lines compared to WT. (B) Tuber weight was indicated for *StGA3ox3*-AS lines in comparison with WT. (C) Representative images for the tubers of *StGA3ox3*-AS lines were obtained. Each image contains tubers from 6 plants per line. (D) Tuber weight for OE lines is indicated. Data was taken from soil grown plants after a one month of SD induction, and plotted from 10 individual plants per line (mean $\pm$ SEM; n=10) (E) Representative images for the tubers were obtained. Each image contains tubers from 12 plants per line. Scale bar= 1 cm. Asterisks (one and two) indicate significant differences at $p < 0.05$, $p < 0.01$, respectively, using one way ANOVA. ns= not significant at < 0.05 .

Grafting experiment was carried out to test if leaf morphology or other pleiotropic phenotypes of the transgenic lines have any effect on tuberization of transgenic lines (Fig. 3.13 A-B). We observed no changes in tuber numbers for OE/OE, WT/OE and OE/WT grafts (Fig.

3.13 C-D). The tuber numbers in hetero-grafts of WT/AS and AS/WT were also comparable to WT/WT homo-grafts, suggesting that an increase of the tuber numbers in *StGA3ox3*-AS lines might not be a result of change in the leaf morphology. Instead, it could be due to the impaired bioactive GA levels.

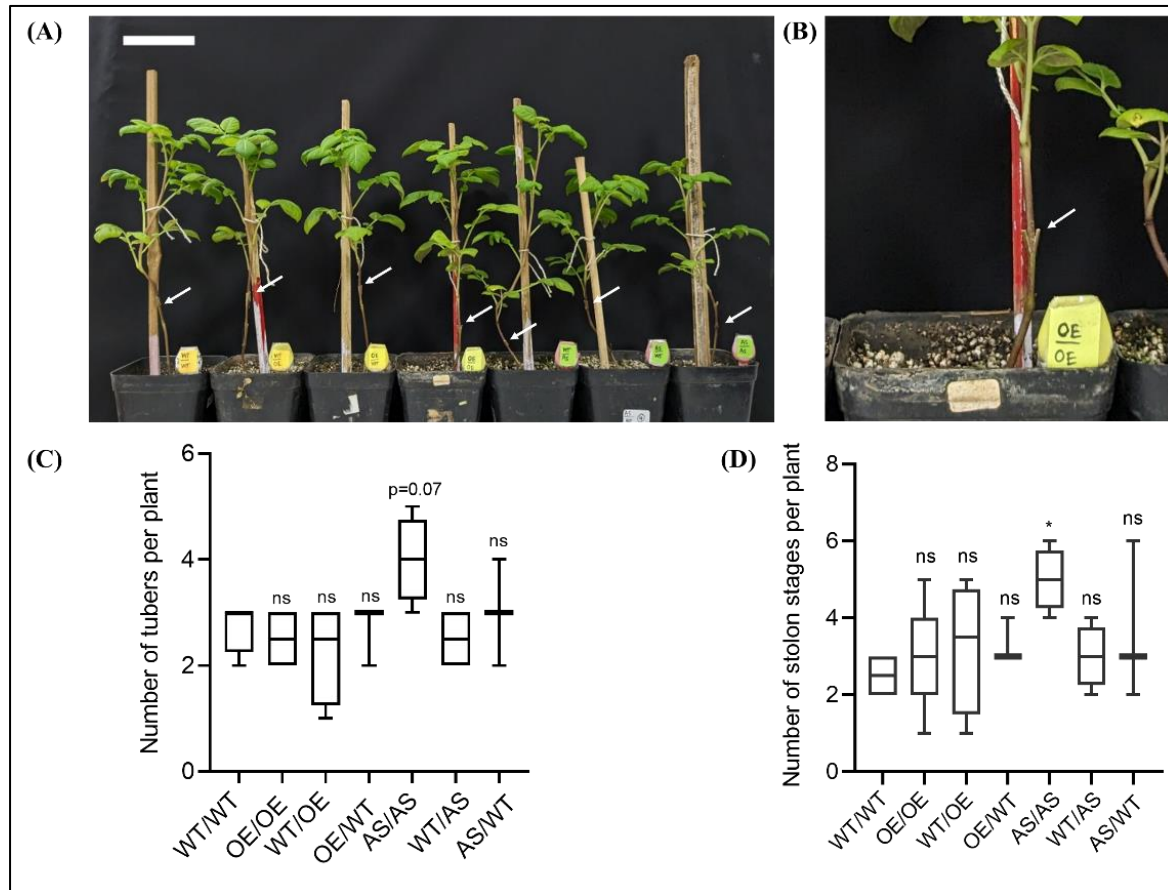


Fig. 3.13 Tuber phenotype in grafts generated from *StGA3ox3* OE, AS lines and WT. (A) Representative images for the graft combinations are given. Scale bar = 10 cm. (B) Zoomed image for graft junction is also represented. White arrows indicate the graft junction. (C) Number of tubers and (D) Total number of stolon stages per plant from various grafts, after 21 days of SD induction. Error bars indicate standard error of means (SEM) from biological replicates. One way ANOVA was performed to check the level of significance. ns = not significant at $p > 0.05$. Data is plotted with $n=5$ for all three types of homo-grafts (WT/WT, OE/OE, AS/AS); $n=4$ for all hetero-grafts (WT/OE, AS/WT, WT/AS) and $n=3$ for OE/WT hetero-graft.

3.3.7 *StRGA4* overexpression lines have high levels of siRD29(-) and mimic the tuber phenotype of *StGA3ox3*-AS lines

Disease resistance genes from the *Solanaceae* family are known to be targeted by miR482/2118 members to produce secondary phasiRNAs (Zhai et al., 2011; Shivaprasad et al., 2012) that further regulate numerous defence-related genes. In absence of any pathogen attack,

miR482 family mediated secondary phasiRNA cascade remains active. After pathogen attack miR482 levels reduces in order to express and maintain high levels of *disease resistance* genes. We utilized the principle of this signaling cascade to overexpress siRD29(-) in potato. To enhance the expression of siRD29(-), constitutive OE lines of *StRGA4* (OE8 and OE10) were generated in andigena plants. These lines exhibited reduced levels of *StRGA4* (Fig. 3.14A) as the transcript is being targeted by increased levels of Stu-miR482c (Fig. 3.14B). Both OE lines showed 2-2.5 fold increase of siRD29(-) levels in the leaves (Fig. 3.14C). The corresponding ~70% decrease in *StGA3ox3* levels was observed (Fig. 3.14D).

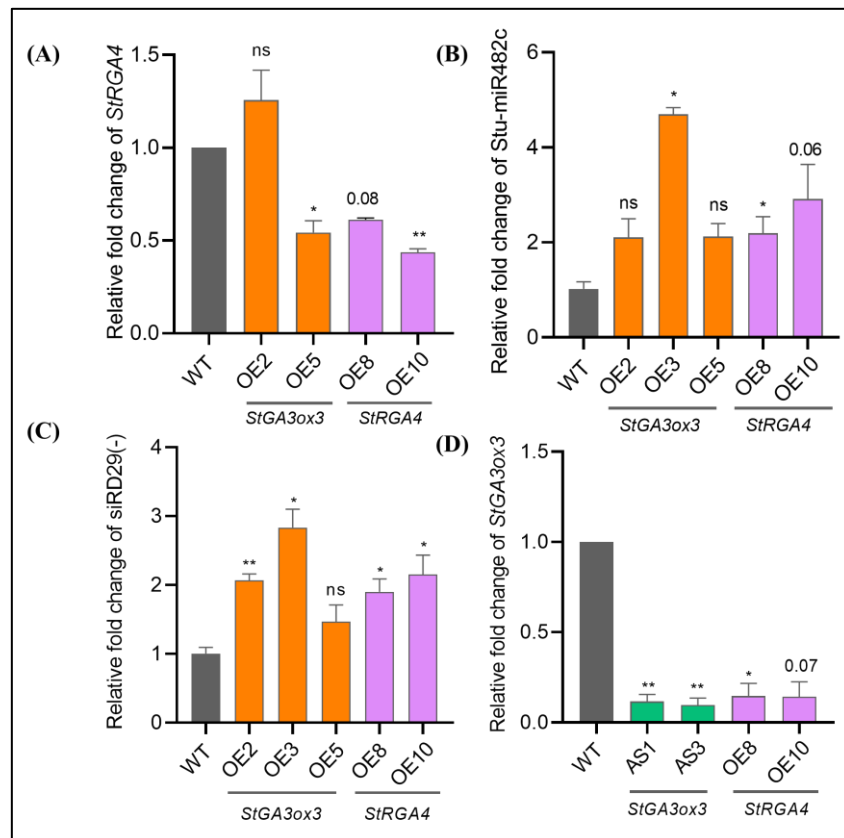


Fig. 3.14 Overexpression lines of *StGA3ox3* and *StRGA4* have enhanced levels of siRD29(-). (A) Expression of the phasiRNA module components, such as *StRGA4* (A), Stu-miR482c (B), and siRD29(-) (C) in leaves of overexpression lines of *StGA3ox3* (OE2, OE3 and OE5) and *StRGA4* (OE8 and OE10) compared to WT. (D) *StGA3ox3* expression in *StRGA4::OE* lines and *StGA3ox3::AS* lines (AS1 and AS3) compared to WT. *StEIF3e* was used for normalizing the gene expression data. Error bars indicate standard error of means (SEM) from three biological replicates (n=3). Asterisks (one and two) indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively, using Student's t-test. ns = not significant at $p > 0.05$.

StRGA4-OE lines mimicked the specific phenotypes of *StGA3ox3-AS* lines, for example reduced plant height (Fig. 3.15 A), no change in the final tuber weight (Fig. 3.15 C-D) but an increased tuber numbers (Fig. 3.15 B, D).

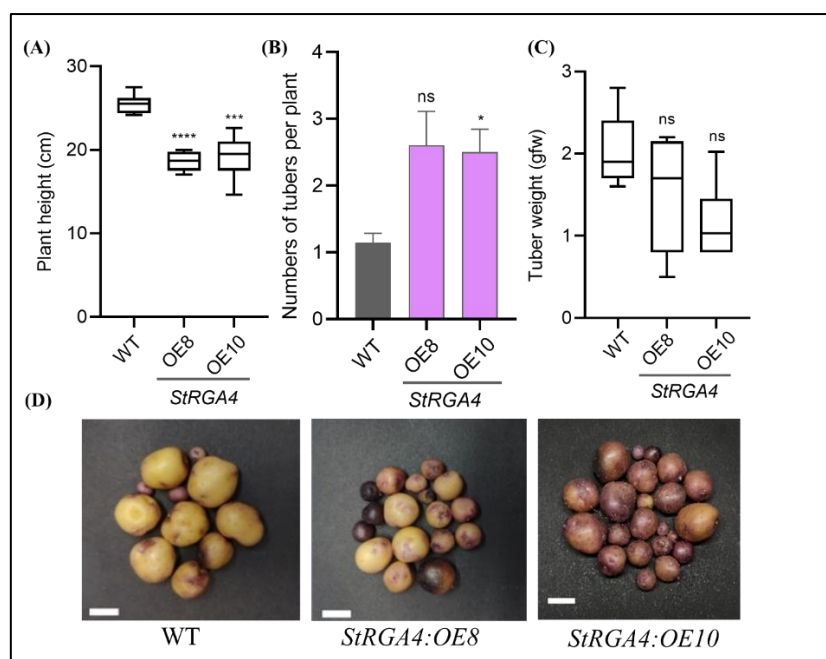


Fig. 3.15 *StGA4*-OE lines exhibits phenotype similar to *StGA3ox3*-AS lines with reduced tuber number. (A) Plant height for *StGA4*-OE lines is shown in compared to WT (n=12). (B) Tuber numbers and (C) Tuber weight in comparison with WT are given. Data from 6 independent plants for each line (n=6) is represented. (G) Each image contains tubers from 6 plants per line. Scale bar= 1 cm. One way ANOVA was performed to check the level of significance. Asterisks (one, three and four) indicate significant differences at $p < 0.05$, $p < 0.005$ and $p < 0.001$, respectively. ns = not significant at $p > 0.05$.

We also quantified module related genes in SD-induced stolons of *StGA4*-OE lines which exhibited increased levels of siRD29(-), followed by a reduction in level of *StGA3ox3* (Fig. 3.16). These results suggest that the gene regulatory module (GRM) is functional during stolon-to-tuber transitions of potato.

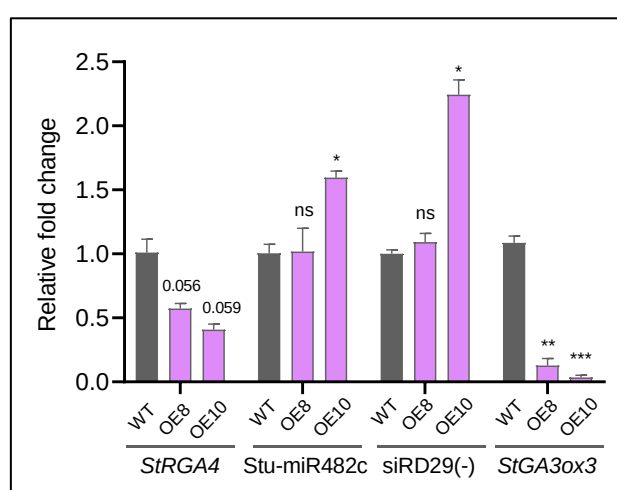


Fig 3.16 *StGA4*-OE lines have enhanced levels of siRD29(-) in stolons. Expression of the module components, *StGA4*, *Stu-miR482c*, *siRD29(-)* and *StGA3ox3* in the SD-induced (four days) stolons of *StGA4* (OE8 and OE10) overexpression lines as compared to WT. Error bars indicate standard error

of means (SEM) from three biological replicates (n=3). One way ANOVA was performed to check the level of significance. Asterisks (one, two and three) indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. ns = not significant at $p > 0.05$.

3.3.8 StGA3ox3 controls tuber-stalk development under *in vitro* conditions

A transient overexpression of *StGA3ox3* in potato leaves led to an increase in GA_1 levels post 48 hrs of agro-infiltration, suggesting that *StGA3ox3* could contribute to GA_1 biosynthesis pathway (Fig. 3.17). Further, to study if *StGA3ox3* influences the earliness for tuber formation, we conducted *in vitro* tuberization assays using OE lines of *StGA3ox3* and *StRGA4*, and *StGA3ox3-AS* lines. *StGA3ox3-OE* exhibited slight delay in tuberization compared to control (Fig. 3.18 A-B), whereas *StGA3ox3-AS* and *StRGA4-OE* lines did not show delay in tuber formation. We observed sessile tuber formation in WT andigena plants. Interestingly, more than 50% tubers of *StGA3ox3-OE* lines showed stalk formation as compared to WT (Fig. 3.19 A), possibly due to increased levels of bioactive GAs in the tuberizing nodes. Consistently, a transient overexpression of *StGA3ox3* in potato leaves led to an increase in GA_1 levels post 48 hrs of agroinfiltration, suggesting that *StGA3ox3* could contribute to GA_1 biosynthesis pathway (Fig. 3.17 A). *StGA3ox3-AS* and *StRGA4-OE* lines exhibited sessile tubers similar to control plants (Fig. 3.19 A-B).

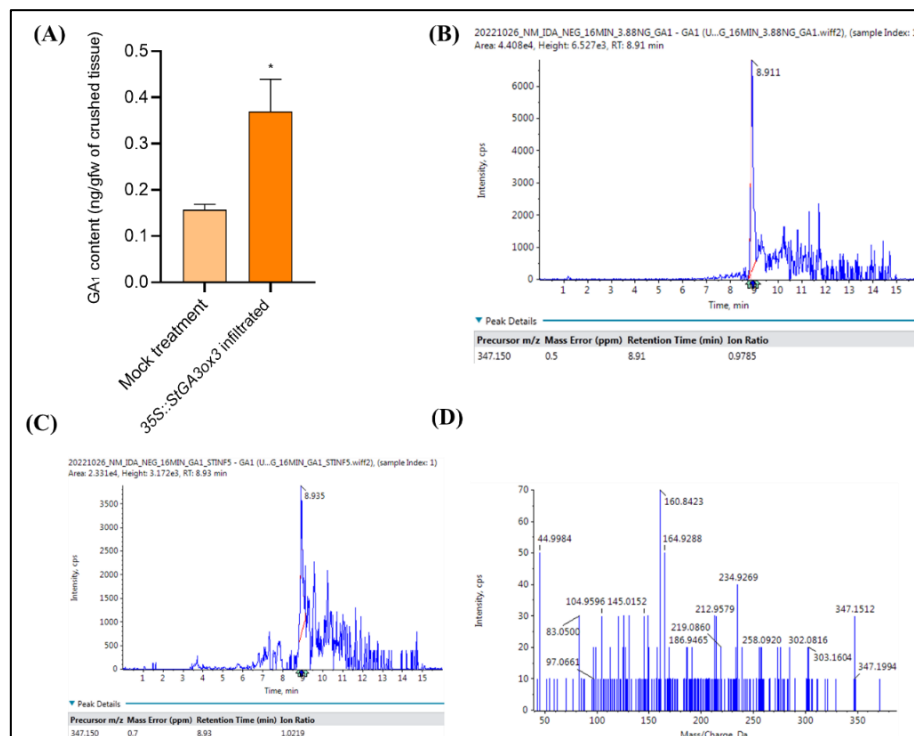


Fig. 3.17 Quantification of GA_1 levels from leaves of potato plant infiltrated with *Agrobacterium* containing *35S::StGA3ox3* gene construct. (A) Quantification of GA_1 levels from leaves of potato

plant infiltrated with *Agrobacterium* containing 35S::*StGA3ox3* gene construct. (B) Chromatogram of GA₁ standard (m/z: 347.15 g/mol; 3.88 ng/ml). (C) Chromatogram of GA₁ from agroinfiltrated leaf sample of potato. (D) MS/MS peaks for GA₁ detected in agroinfiltrated leaf samples.

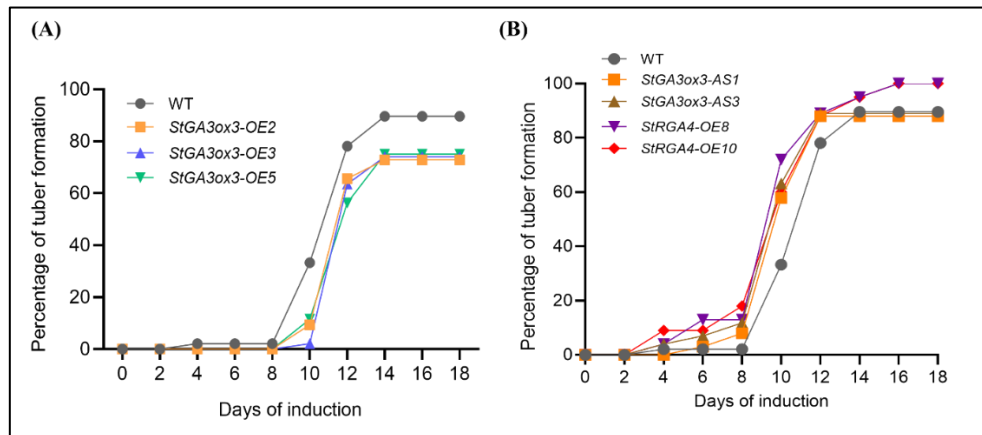


Fig. 3.18 Rate of tuber formation in transgenic lines during *in vitro* tuberization assay. (A) The percentage of tubers produced by three OE lines of *StGA3ox3* (OE2, OE3 and OE5) and (B) *StGA3ox3*-AS, *StRGA4*-OE lines on 8% sucrose in dark under *in vitro* conditions over a period of 18 days. Data is plotted at 2 day intervals with WT.

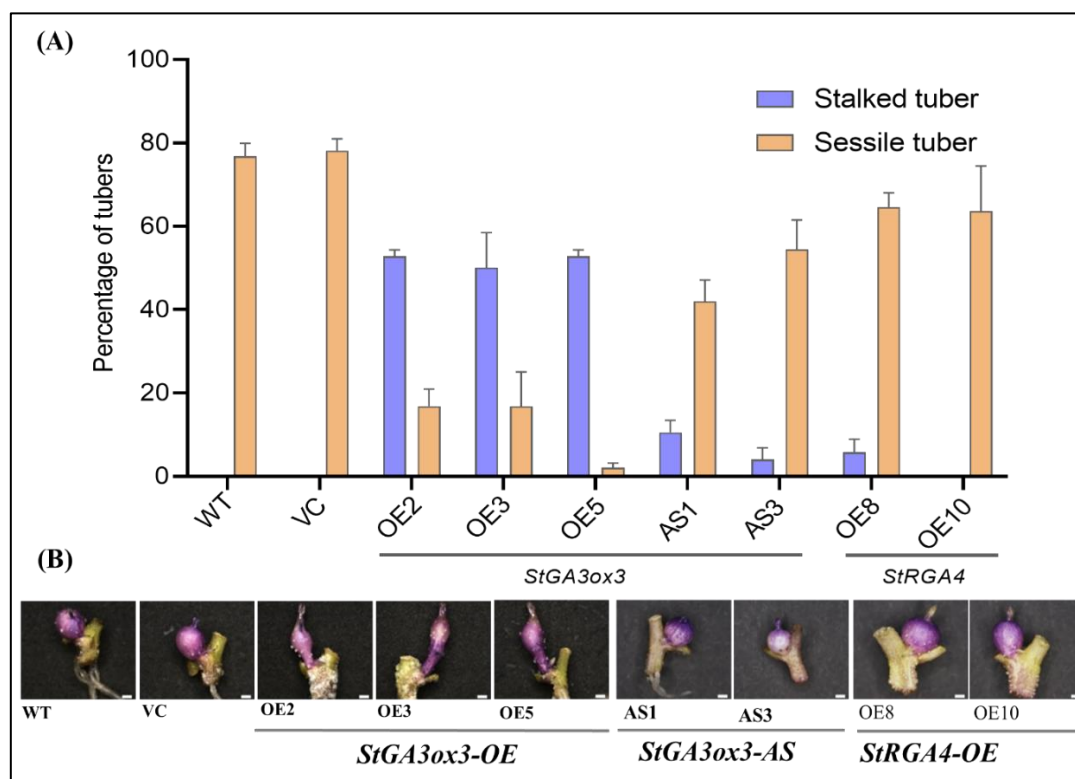


Fig. 3.19 Altered *StGA3ox3* expression influences development of tuber-stalk during *in vitro* tuberization assays. (A) *In vitro* tuberization assay. Single node explants from overexpression lines of *StGA3ox3* (OE2, OE3 and OE5) and *StRGA4* (OE8 and OE10), and the antisense lines of *StGA3ox3*

(AS1 and AS3) along with WT and VC plants were grown on 8% sucrose in dark. Percentage of tubers formed in each line were scored on alternate days until 12th day, and the final tuber number percent is plotted. (B) The nodes with sessile versus stalked-tubers were noted during the observations. Mostly, OE lines of *StGA3ox3* produced stalked-tubers, whereas antisense lines of *StGA3ox3* or overexpression lines of *StRGA4* showed mostly sessile tubers.

Further, addition of paclobutrazol (GA inhibitor) to the tuber-inducing media (8% sucrose) resulted in the formation of sessile tubers in *StGA3ox3*-OE lines similar to WT (Fig. 3.20 A-B). To test whether *StGA3ox3* can control tuber-stalk development *in vivo* (in soil), we measured the lengths of the stolons produced by the transgenic lines. *StGA3ox3*-OE lines (#OE2 and #OE3) exhibited higher stolon lengths compared to WT, whereas the stolon lengths in *StGA3ox3*-AS and *StRGA4*-OE lines did not vary significantly (Fig. 3.21 A-B). We did not observe a significant increase in the stolon length of *StGA3ox3* #OE5 line. This may be due to the fact that independent OE lines do not always show similar phenotypes because of the variations in the level of the transgene expression. Our results indicate that *StGA3ox3* controls the tuber-stalk development in soil grown plants, similar to the *in vitro* tuberization assays.

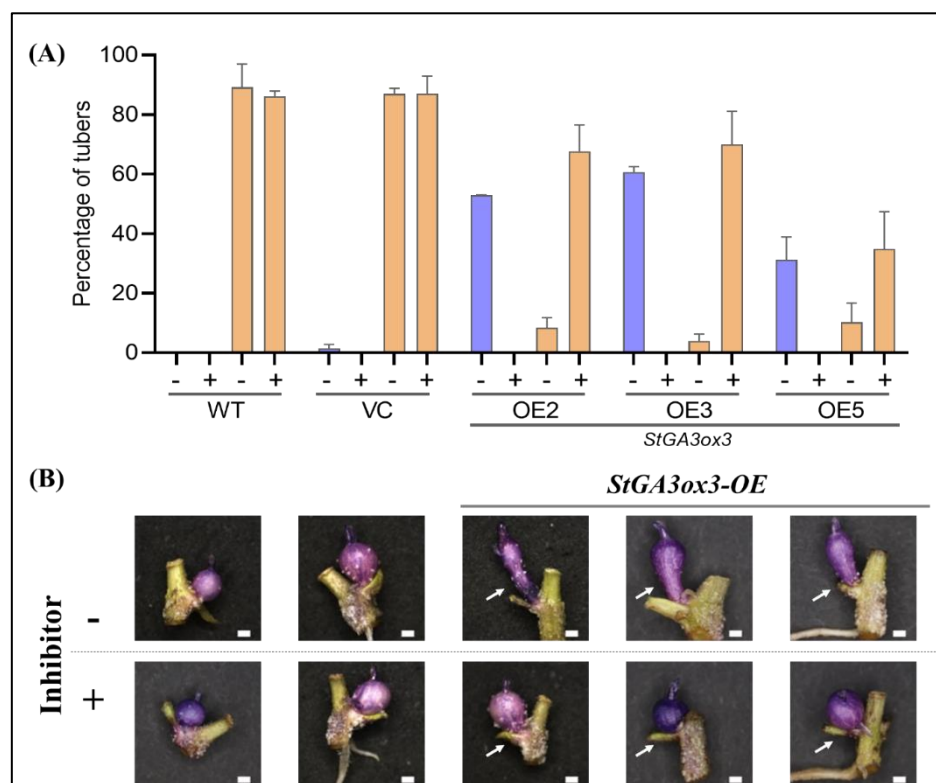


Fig. 3.20 Effect of GA inhibitor treatment on *StGA3ox3*-OE lines during *in vitro* tuberization assay. (A) *In vitro* tuberization assay using three types of transgenic lines with supplementation of a GA biosynthesis inhibitor, paclobutrazol (0.5 mg/L). The symbols (+ and –) indicate the presence or absence of GA inhibitor in the MS media. (B) Representative images of each type of node with tuber are given (Scale bar=1 mm).

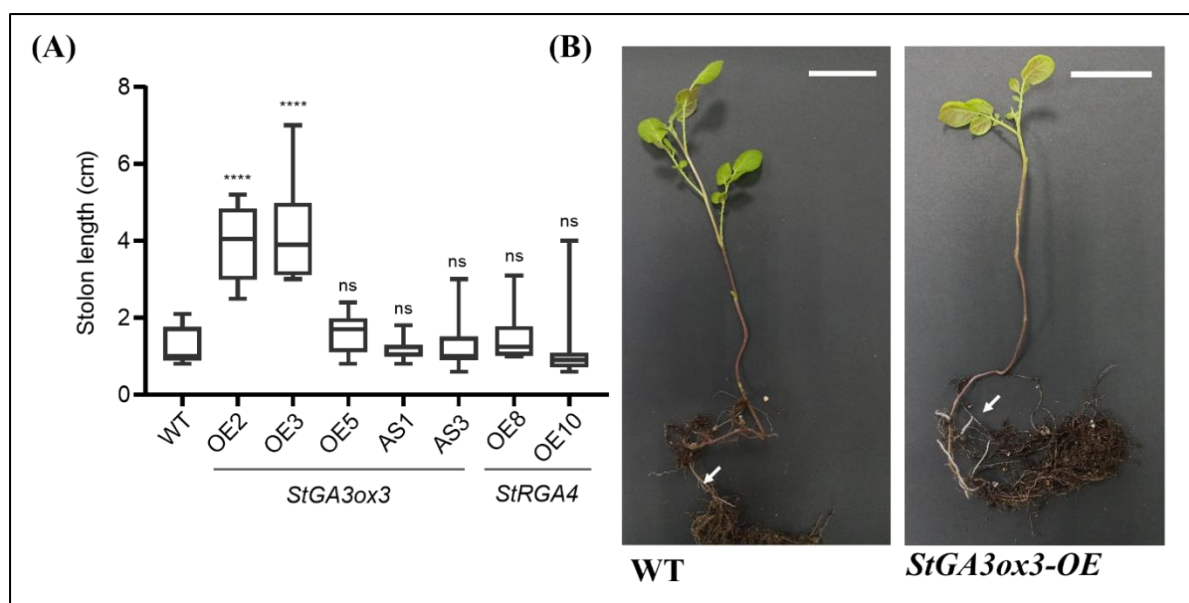


Fig. 3.21 Effect of *StGA3ox3* expression on stolon length in soil-grown plants. (A) Stolon lengths were measured in eight-week old soil grown transgenic lines, subjected to SD photoperiod for four days. Data is from 12 independent plants for each line (n=12) is represented. Asterisks (****) indicate significant differences at $p < 0.0001$ using one way ANOVA. ns= not significant at < 0.05 . (B) Representative plant images for stolon length are shown. Scale bar=10cm.

3.3.9 Altered expression of *StGA3ox3* affect GA levels in transgenic lines

To investigate the effect of altered *StGA3ox3* expression on the GA contents, we quantified five gibberellins (three bioactive GAs: GA₃, GA₄ and GA₇, one GA precursor: GA₁₂, and one GA degradation product: GA₈) from the shoot apices of transgenic lines (Table 3.6). Levels of GA₁₂ precursor were increased in *StGA3ox3*-AS and *StRGA4*-OE lines. It is known that during GA biosynthesis, GA₁₂ can either follow 13-hydroxylation or non-13-hydroxylation pathway to generate GA₁, GA₄, GA₃ and GA₇ respectively. The levels of bioactive GA₃ were higher in *StGA3ox3*-OE line (# OE3). OE line (#2) though showed increase in GA₃ content but it was below the significant threshold. However, *StGA3ox3*-AS lines did not exhibit a change in GA₃ levels. GA₃ levels were significantly less in both of the *StRGA4*-OE lines. *StGA3ox3*-OE (#OE3) and *StRGA4*-OE (#OE8) lines had higher contents of bioactive GA₄ and GA₇. We could not determine GA₁ and GA₂₀ precursors from the shoot-apices. Interestingly, all lines indicated an increase in GA₁ degradation product, GA₈. Particularly, *StRGA4*-OE showed the highest GA₈ content. Gibberellins were also measured from stolons of transgenic lines, but no GA peaks were detected in any of the samples, except for *StGA3ox3*-OE lines, which exhibited low levels of bioactive GAs and higher amount of GA₈.

Table 3.3 Levels of GAs (ng/g of freeze dried tissue weight) in the shoot-apices of the transgenic lines (*StGA3ox3-OE*, *StGA3ox3-AS* and *StRGA4-OE*) compared to wild-type (WT) and digena plants after six weeks in long-day conditions. GAs were quantified using LC-MS/MS with internal standards. The values are means of three independent biological replicates (n= 3) $\pm$ standard error of means (SEM). One way ANOVA was performed to check the level of significance. Asterisks (one, two, three and four) in parenthesis indicate significant differences at $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

		GA concentrations in shoot-apices (ng/g of dried weight) represented as Mean $\pm$ SEM				
		GA3	GA4	GA7	GA8	GA12
Wild-type	WT	154.9 $\pm$ 24.2	1132.9 $\pm$ 116.1	16395.7 $\pm$ 1073.3	306.5 $\pm$ 26.2	1052.0 $\pm$ 76.7
<i>StGA3ox3-OE</i>	OE2	384.4 $\pm$ 169.7	1387.8 $\pm$ 294.4	13936.4 $\pm$ 1485.3	385.8 $\pm$ 71.0	1055.2 $\pm$ 107.5
	OE3	561.7 $\pm$ 63.9(*)	1783.0 $\pm$ 186.4	24926.9 $\pm$ 2163.9(*)	496.3 $\pm$ 67.7	1435.7 $\pm$ 67.0
<i>StGA3ox3-AS</i>	AS1	200.7 $\pm$ 26.3	1517.1 $\pm$ 100.1	23570.5 $\pm$ 2170.2	421.6 $\pm$ 23.4	1336.6 $\pm$ 86.8
	AS3	224.3 $\pm$ 30.9	1535.9 $\pm$ 48.4	22428.7 $\pm$ 565.0	422.0 $\pm$ 38.1	1422.7 $\pm$ 72.2
<i>StRGA4-OE</i>	OE8	57.2 $\pm$ 3.2(****)	2187.2 $\pm$ 490.0(*)	64234.3 $\pm$ 8146.4(***)	1198.7 $\pm$ 101.5(**)	1968.1 $\pm$ 198.7(***)
	OE10	66.6 $\pm$ 10.3(****)	1090.1 $\pm$ 172.6	42182.6 $\pm$ 3551.5(**)	1105.8 $\pm$ 63.9(**)	1996.6 $\pm$ 104.3(***)

3.3.10 Promoter of *StGA3ox3* gene has phytohormone responsive motifs.

The GUS activity in *promStGA3ox3::GUS* lines was highest in swollen stolons compared to SD induced stolon. These results suggested that *StGA3ox3* expression in stolons could be regulated through its promoter. The 2.2 kb promoter sequence of *StGA3ox3* was analysed for the presence of *cis*-regulatory motifs using tools such as PLANTPAN3.0 and PLACE (Chow et al., 2019; Higo et al., 1999). Several motifs were identified, such as bHLH (59), AP2-ERF (200), bZIP/HD-ZIP (94), MYB (21), MYB/SANT (65) WRKY (253), DOF (356), homeodomain TFs (90), ZF-HD (85), NAC/NAM (33), etc. (Fig. 3.22 A). To analyse effects of phytohormones on *StGA3ox3* promoter activity, we performed qRT-PCR analysis for *PromstGA3ox3::GUS* lines treated with Jasmonic acid (JA) and Salicylic acid (SA) respectively. JA induced GUS expression in lines (Fig. 3.22 B) whereas SA did not have any effect on *StGA3ox3* promoter activity (Fig. 3.22 C).

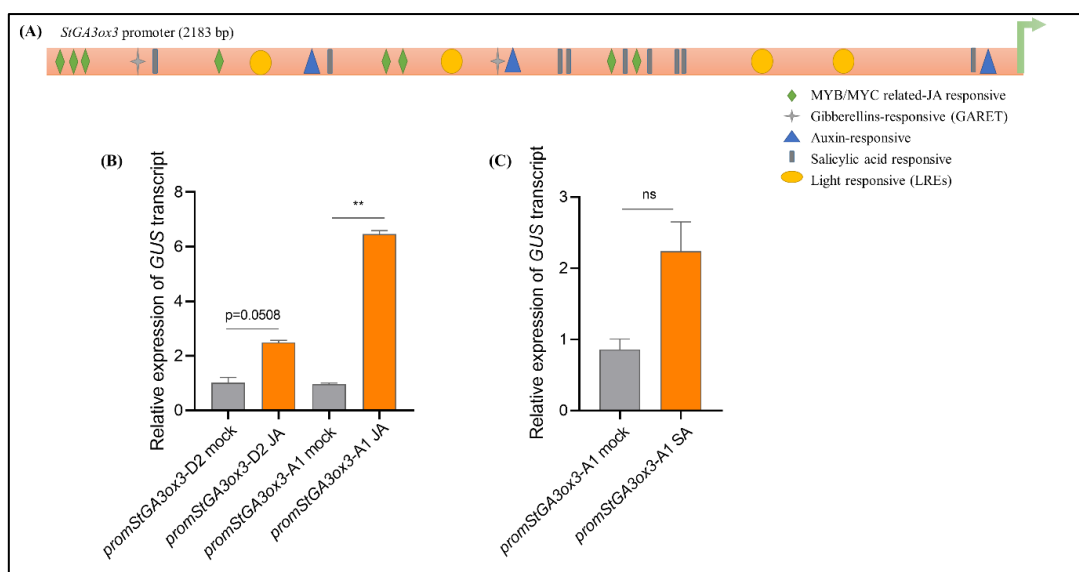


Fig. 3.22 *StGA3ox3* promoter is enriched with hormone inducible motifs. (A) Schematics of *StGA3ox3* promoter highlighting the presence of hormone inducible motifs. Green arrow indicated transcription start site (TSS). Expression of GUS transcript in the leaves of *promStGA3ox3::GUS* lines, 48 hrs post treatment of hormones (B) Jasmonic acid and (C) Salicylic acid. D2= *promStGA3ox3::GUS* in *desiree*; A1= *promStGA3ox3::GUS* in *andigena*. One way ANOVA was performed to check the level of significance. ** p<0.01, ns=not significant at <0.05.

Further, the effect of phyto-hormones on the expression of *StGA3ox3* was tested *in vivo*, JA, induced *StGA3ox3* expression while SA treatment did not have significant effect. Gibberellic acid (GA₃) and Auxin (2, 4-D) treatment to WT potato plants resulted in a decrease in *StGA3ox3* levels in leaves.

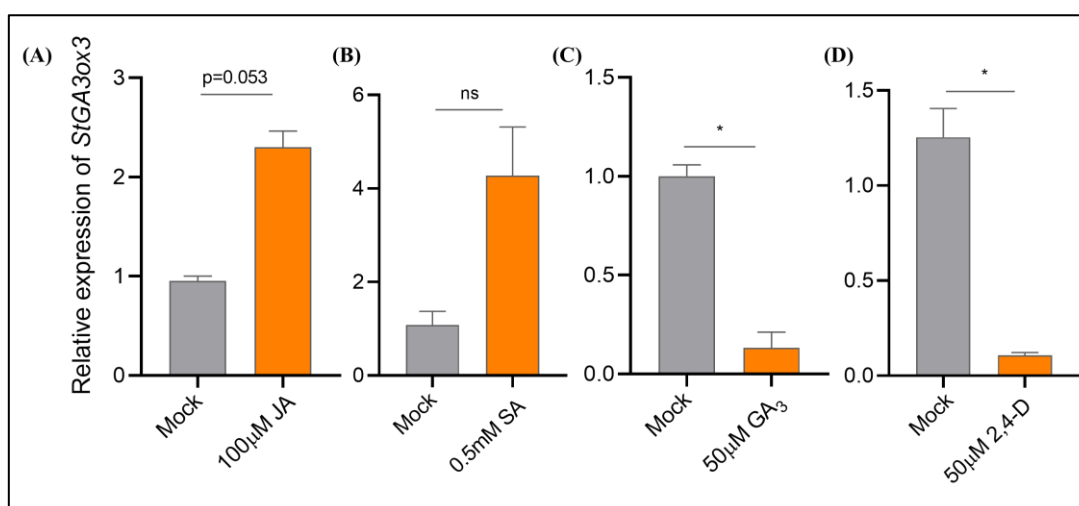


Fig. 3.23 Effects of phyto-hormone treatments on *StGA3ox3* expression. *StGA3ox3* transcript levels in the local leaves of WT potato plants, harvested 48hr post treatment with (A) Jasmonic acid (JA), (B) Salicylic acid (SA), (C) Gibberellin (GA₃) and (D) Auxin (2, 4-D). Student's t- test was performed to check the level of significance. * p<0.05, ns=not significant at >0.05.

3.3.11 StBEL5 (a BEL-like TF) induces *StGA3ox3* expression in leaves of *promStGA3ox3::GUS* lines

Interestingly, 12 tandem TGAC core motifs were also identified in *StGA3ox3* promoter using RSAT tool (Helden et al., 2000) (Fig. 3.25 A). Among them, six TGAC core motifs were separated by a lesser than 40 bp linkers (Fig. 3.25 A; Table 3.6).

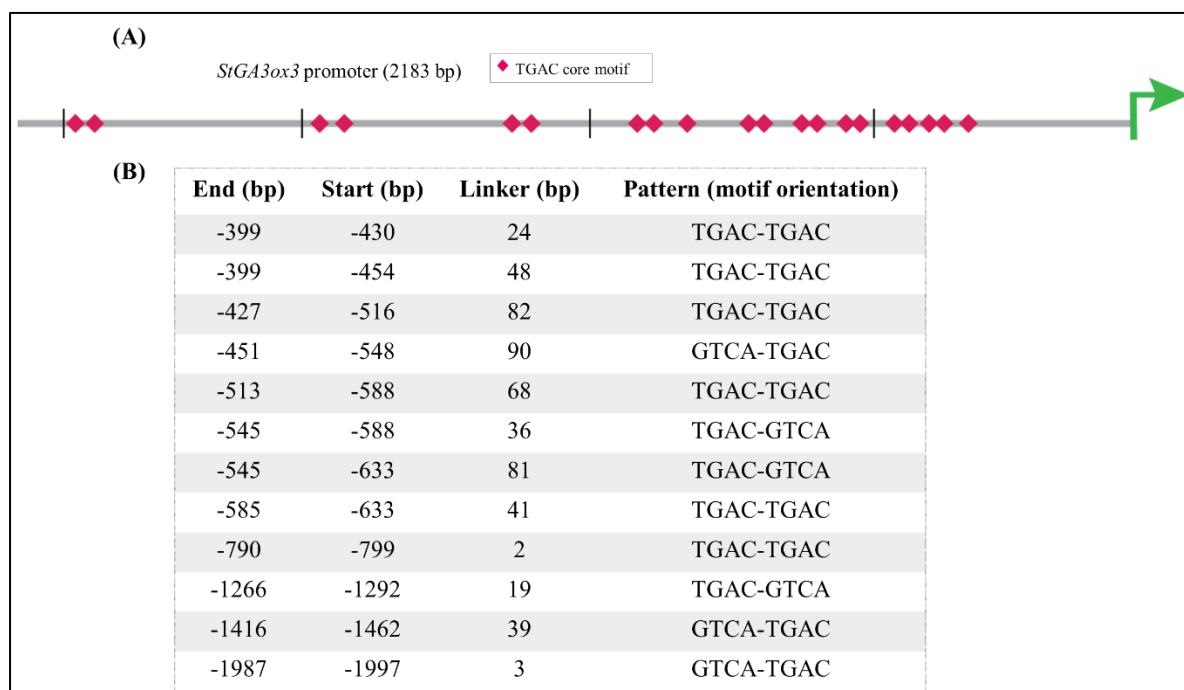


Fig. 3.24 *StGA3ox3* promoter is enriched with StBEL5 binding site motifs. (A) Schematics of *StGA3ox3* promoter highlighting the presence of TGAC core motifs. The promoter sequence (~2.1 kb) characterized, was used to identify the TGAC core motifs (denoted by pink diamond). (B) The detailed location of each motif is given also in Table 3.5. TSS= transcription start site.

To study this regulation, we agroinfiltrated *StBEL5-OE* gene construct in the leaves of *promStGA3ox3::GUS* lines. As *StBEL5* expression is known to be wound-inducible (Chatterjee et al., 2007), we observed an increased expression of *StBEL5* as well as *GUS* in the mock (control) compared to non-infiltrated leaves. Quantitative PCR from *StBEL5* infiltrated leaves showed an increased *GUS* expression (Fig. 3.25 A-B). *GUS* protein activity was also increased in *StBEL5-OE* gene construct infiltrated leaves (Fig. 3.25 C), suggesting that *StBEL5* induces the expression of *StGA3ox3* in potato.

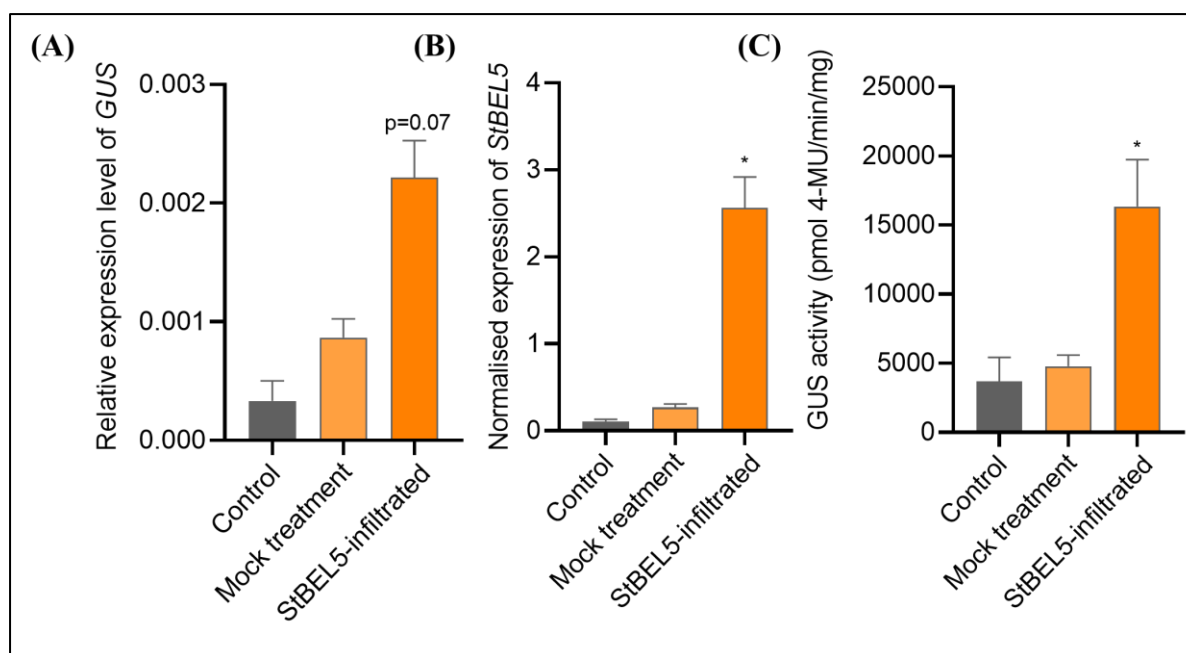


Fig 3.25 BEL1-like TF StBEL5 regulates the expression of *StGA3ox3*. Transcript abundance of (A) *GUS* and (B) *StBEL5* in the leaves of *promStGA3ox3::GUS* line agroinfiltrated with *35S::StBEL5* gene construct. (C) Quantification of GUS activity in leaves of *promStGA3ox3::GUS* line agroinfiltrated with *35S::StBEL5* gene construct. Student's t test was performed to check the level of significance. * $p < 0.05$, ns=not significant at > 0.05 .

3.4 Discussion

Small RNA (sRNA) regulatory networks orchestrate various plant developmental processes, such as initiation of SAM and its maintenance, leaf polarity establishment, flowering time, and root development (Dong et al., 2022; Liu et al., 2022). Tuberization in potato is governed by numerous mobile signals, transcription factors and phytohormones. These factors co-ordinate a series of developmental events in the stolon to form a mature tuber. However, if sRNA networks could regulate stolon-to-tuber transitions in potato is not investigated. We have earlier reported that a number of sRNA candidates could potentially be involved in early stages of tuber development (Kondhare et al., 2018, 2021). Here, we demonstrate the functionality of a novel gene regulatory module (GRM), *Stu-miR482c-StGA4-siRD29(-)-StGA3ox3* and its significance during stolon-to-tuber transitions in potato.

3.4.1 Novel *Stu-miR482c-StGA4-siRD29(-)-StGA3ox3* GRM is functional in potato

In this study, we provided the experimental evidences for a novel phasiRNA regulating a GA biosynthesis gene, *StGA3ox3* in potato. Through transient expression assays in *N. benthamiana*, followed by small RNA-sequencing, we showed that *Stu-miR482c* triggers

synthesis of 21-nt phasiRNAs from the *StRGA4 PHAS* locus. Further, we demonstrated that one of the phasiRNAs from this locus, siRD29(-) is generated at 29th position from the antisense strand in a RDR6-dependent manner, and cleaves *StGA3ox3* transcript *in planta* (Figs. 3.1-3.5). These findings establish the fact that *Stu-miR482c-StRGA4-siRD29(-)-StGA3ox3* GRM is active. SiRD29(-) is found to be abundant in leaves under LD and was highest in SD-induced stolons (Fig. 3.4). Interestingly, siRD29(-) and *StGA3ox3* transcript levels exhibited an inverse expression patterns during stolon to swollen stolon stage under SD conditions (Fig. 3.4 C; 3.6 D), indicating the possible role of this module in tuber development.

Earlier, two isoforms of GA3ox (referred as *StGA3ox1* and *StGA3ox2*) were reported in potato (Bou-Torrent et al., 2011; Roumeliotis et al., 2013a). However, only *StGA3ox2* has been studied for its role in tuberization (Bou-Torrent et al., 2011; Roumeliotis et al., 2013a). Here, we have identified another isoform of StGA3ox (referred as *StGA3ox3*) (Fig. 3.2) and it is a target of siRD29(-) (Fig. 1E). Unique expression patterns of *StGA3ox3* (Fig.3.3) suggests that it could have a distinct role in plant growth and development. Previously, *StGA3ox2* expression was found to be highly reduced in stolons under SD conditions (Bou-Torrent et al., 2011). Surprisingly, we observed the highest expression of *StGA3ox3* in SD-induced swollen stolons (Fig. 3.6 D). It raises an important question: what is the relevance of increased *StGA3ox3* expression during the transitions of a stolon to swollen stolon? Reduced GA level is a pre-requisite for tuber development. This perhaps necessitates a regulatory mechanism that could control *StGA3ox3* levels during stolon-to-tuber transitions in potato. For example, in rice, miR9678 has been shown to trigger phasiRNA production from long non-coding RNA WSGAR that could target GA biosynthesis genes to delay seed germination (Guo et al., 2018), suggesting a possible regulation of GA metabolism through phasiRNAs in plants.

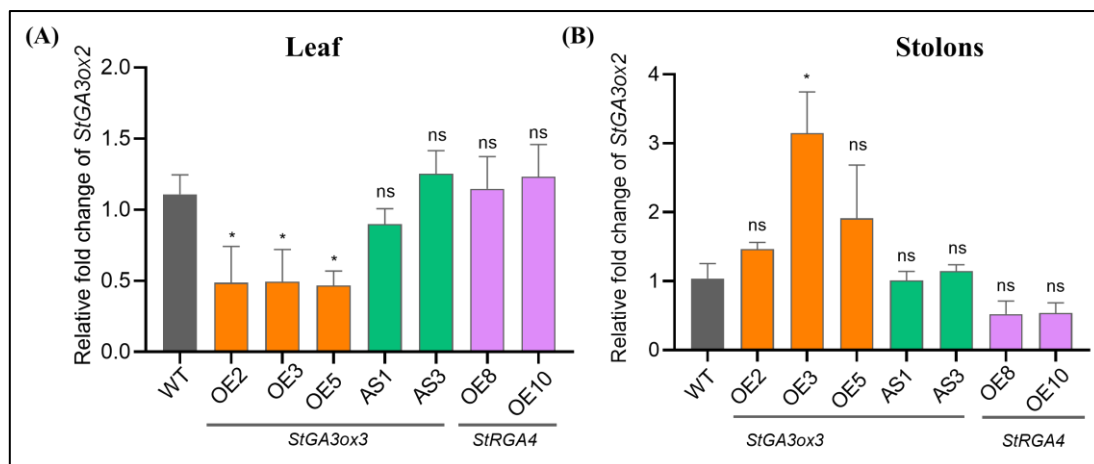


Fig. 3.26 Expression of *StGA3ox2* transcript in *StGA3ox3* OE, AS and *StRGA4* OE lines. Transcript levels of *StGA3ox2* in (A) leaves and (B) SD-induced stolons of *StGA3ox3*-OE, *StRGA4*-OE and

StGA3ox3-AS lines as compared to WT. *StEIF3e* was used as reference gene expression analysis. Error bars indicate standard error of means (SEM) from three biological replicates (n=3). One way ANOVA was performed to check the level of significance. Asterisks (one, two and three) indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. ns = not significant at $p > 0.05$. For stolon tissues, eight week soil grown transgenic plants were subjected to SD photoperiod for four days. Leaf and stolon samples were harvested between Zeitgeber time ZT = 2 and ZT = 4

Bioactive GAs control leaf expansion and the elongation of stem and root tissues (Davies, 2004). We noted that the OE and AS lines of *StGA3ox3* exhibited typical GA-mediated phenotypes (Fig. 3.9- 3.11). Interestingly, *StGA3ox3-AS* lines showed higher tuber numbers compared to WT (Fig. 3.12 A-B). Previously, a report by Roumeliotis et al. (2013a) showed that *StGA3ox2* RNAi lines had increased tuber numbers (per plant) possibly due to altered GA:auxin ratio in stolons. However, we did not observe any change in the expression of *StGA3ox2* in *StGA3ox3-AS* lines (Fig. 3.26), suggesting that the phenotypes we observed were specifically due to the knockdown of *StGA3ox3* only. To identify if pleiotropic phenotypes of *StGA3ox3-OE* lines have any effect on tuber numbers, grafting was conducted. Heterografts (OE/WT, WT/OE) did not show any change in the tuber numbers, suggesting that the leaf morphology of *StGA3ox3-OE* lines may not have any direct effect on the tuberization. However, the unaltered tuber numbers in the heterografts of WT/AS could be the effect of transport of bioactive GAs from leaf to stock. Further, we observed that *StRGA4-OE* lines exhibited an increased expression of siRD29(-) in leaves and stolons (Fig. 3.14 C; 3.16) but reduced the levels of *StGA3ox3* transcripts (Fig. 3.14 D; 3.16). These lines had quite similar tuber phenotypes as of *StGA3ox3-AS* lines (Fig. 3.15) suggesting that the Stu-miR482c-*StRGA4*-siRD29(-) signaling cascade is functional and regulates *StGA3ox3* expression in *StRGA4-OE* lines. Hence, it is believable that this GRM is active in leaves as well as in stolons of potato.

Subsequently, we investigated the role of *StGA3ox3* isoform in GA biosynthesis pathway. *GA3ox* gene family catalyzes the final step in the synthesis of bioactive GAs (GA_1 , GA_3 and GA_4) from GA_{20} precursor (Hedden and Phillips, 2000). Previously, *StGA3ox2* was reported to catalyze GA_1 production (Bou-Torrent et al., 2011). Through transient expression in potato (Fig. 3.17 A), we observed an increased GA_1 levels in the leaves infiltrated with *StGA3ox3-OE* construct, whereas GA_3 levels remained unchanged. We noted that the levels of GA_3 and GA_7 were high in *StGA3ox3-OE* lines, whereas *StRGA4-OE* lines had significantly reduced GA_3 levels, indicating that *StGA3ox3* might be involved in GA_3 synthesis (Table 3.6).

GA₃ concentration in AS lines of *StGA3ox3* was similar to WT. Also, the expression of *StGA3ox3* was less in shoot apex. These observations led us to speculate that the functions of *StGA3ox3* in the shoot apex of AS line might be compensated by other GA-biosynthesis enzymes. Further in the constitutive *StRGA4-OE* lines, *StGA3ox3* transcripts are targeted by siRD29(-), hence the effect on GA₃ concentration is quite evident in these lines. Even though GA₁ could not be estimated in samples, but high levels of GA₈ in *StGA3ox3-OE* lines possibly suggests a metabolic turnover resulted from the breakdown of GA₁ produced. These results, in addition to the *StGA3ox3-OE* phenotypes (such as increased plant height, larger leaf size, reduced chlorophyll and anthocyanin content), indicate that *StGA3ox3* isoform could be involved in synthesis of bioactive GA₁ and GA₃.

To dissect the role of *StGA3ox3* in tuber development, we carried out an *in vitro* tuber induction experiment using *StGA3ox3-OE*, *StGA3ox3-AS* and *StRGA4-OE* lines (Fig. 3.18-3.19). As anticipated, *StGA3ox3-OE* lines exhibited delay in tuberization (Fig. 3.18 A), however, noticeable changes were not observed in other transgenic lines. Further, *StGA3ox3-OE* lines produced stalked tubers (Fig. 3.19). During *in vitro* tuberization experiment, application of GA₃ leads to its accumulation in the meristematic region of the nodes that causes cell elongation and formation of stalked tubers (Shibaoka, 1993; Sanz et al., 1996; Xu et al., 1998a). Interestingly, application of paclobutrazol, a GA inhibitor to *StGA3ox3-OE* lines led to the formation of sessile tubers under tuber-inducing *in vitro* conditions (Fig. 3.20), suggesting that enhanced levels of bioactive GA might have stimulated the stalked tuber phenotype. The increased stolon length in *StGA3ox3-OE* lines as compared to WT in soil grown plants (Fig. 3.21) further supports the notion. A recent study by Kou et al. (2021) reported that GA₃ treatment caused elongation in the flower stock in Chinese cabbage *via* induced expansion of cell walls through activation of cell wall proteins like expansins. These results suggest that *StGA3ox3* could be involved in potato tuber-stalk development.

3.4.2 StBEL5 regulates *StGA3ox3* expression in potato

We observed a prominent GUS expression pattern in swollen stolons of *StGA3ox3* promoter lines (Fig. 3.8). This directed us to investigate if any transcriptional regulators govern the expression of *StGA3ox3*. BELs interact with KNOTTED1-LIKE (KNOX) TFs to control diverse aspects of plant growth and development (Hay and Tsiantis, 2010). The BEL-KNOX heterodimers are shown to regulate the expression of key tuberization genes by binding to the tandem TGAC core motifs in their promoters (Chen et al., 2004; Sharma et al., 2016). Presence of 12 tandem TGAC core motifs on the *StGA3ox3* promoter (Fig. 3.24), indicated a possible

regulation by *StBEL5*. Through transient overexpression of *StBEL5* in the leaves of *promStGA3ox3::GUS* lines, we confirm that *StBEL5* acts as an activator of *StGA3ox3* gene expression (Fig. 3.25). Increasing *StGA3ox3* levels by *StBEL5* is counter intuitive, given the fact that GA inhibits tuberization. The key step in the stolon-to-tuber transition is to regulate the GA:auxin ratio to alter cell division pattern (Xu et al., 1998b). Increased auxin at the stolon-tip is transported to the sub-apical region of the stolon upon induction (Roumeliotis et al., 2012a; 2012b), resulting in swelling of the stolon. Thus, it appears that *StBEL5* induces expression of *StGA3ox3* possibly to maintain the GA:auxin ratio in the tuberizing stolons.

3.4.3 Dual regulation of *StGA3ox3* during stolon-to-tuber transitions

Our findings point towards a possible dual regulation of *StGA3ox3* by two independent regulatory pathways- one is through *siRD29(-)* and the other is mediated by *StBEL5*. Obviously, a question arises as to what is the need of such a dual regulation? It is known that at the onset of tuber development, GA levels reduce in the stolon (Xu et al., 1998a). *StBEL5*, a key tuber marker inhibits GA biosynthesis gene *StGA20ox1* (Chen et al., 2004), but upregulates GA catabolism gene *StGA2ox1* (Sharma et al., 2016) to reduce GAs in the stolon. In the present investigation, we found that *siRD29(-)* levels are high in SD-induced stolons of wild-type andigena plants (Fig. 3.4 D). Further, *StRGA4-OE* lines exhibited 2-fold increase in *siRD29(-)* levels and showed phenotypes similar to *StGA3ox3-AS* lines, suggesting that enhanced *siRD29(-)* levels may exert a control to reduce *StGA3ox3* abundance in the SD-induced stolon (Fig. 3.14 D; Fig. 3.16). However, we found induced expression of *StGA3ox3* in swollen stolons, implicating that the control exerted by *siRD29(-)* to reduce *StGA3ox3* levels is possibly overridden by other gene regulatory mechanism in the swollen stolon. We noticed that a tuberization activator *StBEL5* and *StGA3ox3* are abundantly expressed in the swollen stolon (Fig. 3.4 C; Fig. 3.7), suggesting a possible regulation of *StGA3ox3* by *StBEL5*. Indeed, we showed that *StBEL5* increases *StGA3ox3* expression in potato (Fig. 3.25 B-C). As optimal GA levels are required for stolon-to-tuber transitions, it emphasizes the need for a distinct mechanism for spatio-temporal control of GA levels in the stolon, which is possibly achieved either independently by *siRD29(-)* and *StBEL5* or in combination. The identification of such gene regulatory module to regulate an important phytohormone GA, suggests that it is crucial for a plant to make developmental decisions to achieve the ultimate outcome. Our current study proposes a novel GRM that could regulate potato tuber development. What signals activates this module is an interesting question. Moreover, if there is any feedback mechanism from the bioactive GAs to maintain the phytohormone ratios in stolons is not yet investigated.

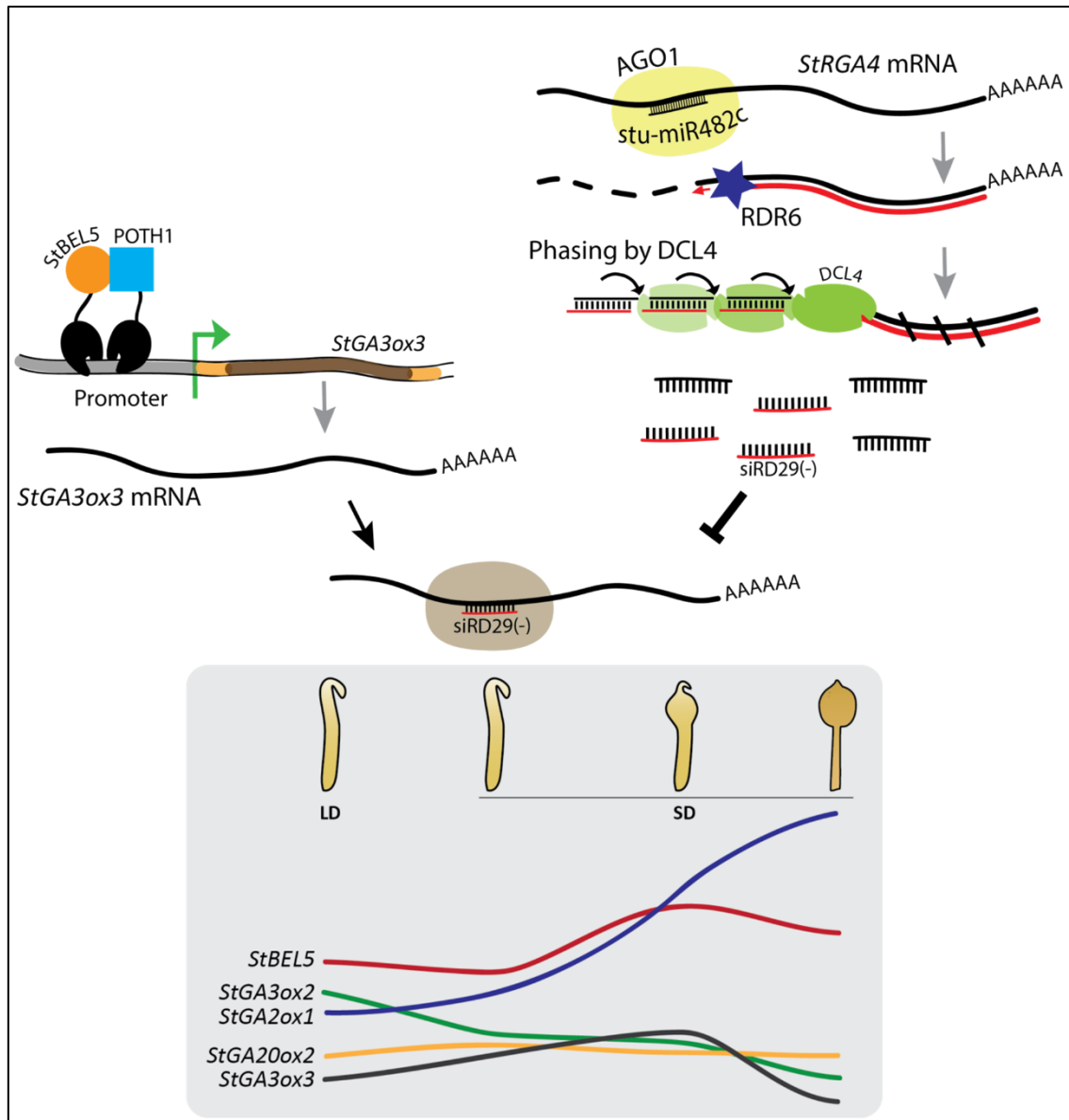


Fig. 3.27 Proposed model for the dual regulation of *StGA3ox3* during stolon-to-tuber transitions of potato. Proposed model for the dual (transcriptional and post-transcriptional) regulation of *StGA3ox3* during stolon-to-tuber transitions of potato. To highlight the role of *StBEL5* and GA metabolism genes, the expression pattern of *StBEL5*, GA biosynthesis genes (*StGA3ox2*, *StGA3ox3*, *StGA20ox2*), and the GA catabolism gene *StGA2ox1* are also shown.

Notably, we show that this GRM is conserved in tomato (*S. lycopersicum*) (Fig. 3.28), indicating the significance of such modules in developmental processes. In conclusion, we show that a novel regulatory network of *Stu-miR482c-StGA4-SiRD29(-)-StGA3ox3* is functional and maintain *StGA3ox3* levels during stolon to swollen stolon transition.

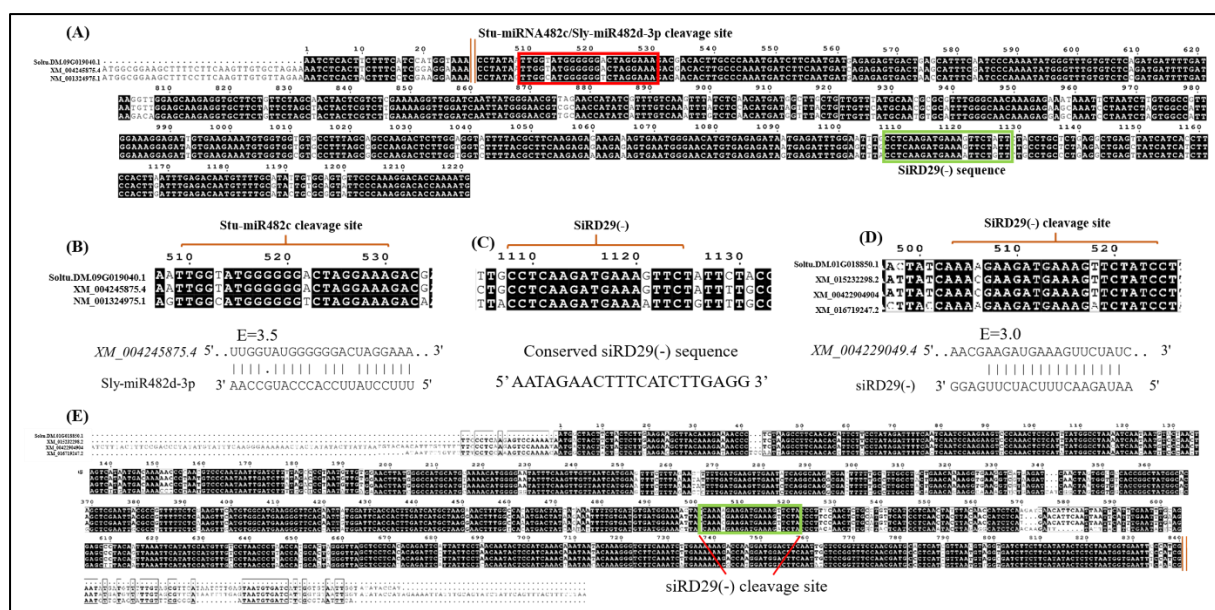


Fig. 3.28 Conservation of stu-miR482c-*StGA4*-siRD29(-)-*StGA3ox3* gene regulatory module in tomato. (A) Alignment for a *PHAS* region from *StGA4* transcripts from potato (Soltu.DM.09G019040.1) and tomato cultivars- *Solanum lycopersicum* (XM_004245875.4) and *Solanum penelli* (NM_001324975.1) is represented. NCBI accessions for sequences are written before the respective sequence used in the alignment study. The Stu-miR482c/Sly-miR482d-3p cleavage site on all transcripts is highlighted in red boxes. Whereas, siRD29(-) location is shown by a green box. (B) Magnified view of the miRNA cleavage site on *StGA4* transcript from alignment. psRNA target prediction represents Sly-miR482d-3p cleaves tomato *SIRGA4*. (C) Conserved siRD29(-) and its phase from aligned sequences are shown. (D) SiRD29(-) cleavage site on aligned *GA3ox3* sequences from potato, tomato and capsicum plants. E= cleavage expectancy from software. (E) Alignment of potato *StGA3ox3* transcript (Soltu.DM.01G018850.1) with *GA3ox3* sequences from tomato (*Sly*, XM_015232298.2; *Sp*, XM_00422904904) and *Capsicum annum* (XM_016719247.2). NCBI accessions for sequences are mentioned. SiRD29(-) cleavage site is highlighted in green box.

In this work, we demonstrate a phased siRNA-mediated regulation of the gibberellin (GA) biosynthesis gene - *StGA3ox3* during tuber development in potato. *StGA3ox3* gene and its regulation have not been previously explored. Through transient expression assays and small RNA sequencing, we establish that Stu-miR482c-*StGA4*-siRD29(-) gene regulatory module (GRM) is active *in planta*. This GRM targets *StGA3ox3* in potato, and we show that *StGA3ox3* is involved in GA₁ and GA₃ biosynthesis. Overexpression of siRD29(-) and knockdown of *StGA3ox3* showed similar tuber phenotypes, indicating the functionality of this module during tuber development. *StGA3ox3* seems to regulate tuber-stalk development. We also demonstrate that *StGA3ox3* is regulated by two distinct mechanisms- a phasiRNA siRD29(-) and a well-known mobile tuberization signal, StBEL5 in potato. Altogether, this module

provides flexibility to the regulation of bioactive GA levels in fine-tuning stolon-to-tuber development process in potato.

3.5 Availability of raw sequencing data

The raw sequencing data were deposited in the NCBI Short Read Archive (SRA) database (<http://www.ncbi.nlm.nih.gov/sra/>) and can be retrieved using the accession number PRJNA931820

Table 3.4 Small RNA sequencing of stolon-to-tuber transitions stages from andigena and identification of *TAS-like* loci - *StRGA4*. The list is generated based on our previous report (Kondhare *et al.*, 2018). One of the siRNAs from *TAS* locus (Chr09:46314301..46314552) highlighted in yellow is referred as siRD29(-) referred in this study.

Sr. No.	TAS genomic location	siRNAs generated from <i>TAS-like</i> loci	siRNA abundance values
1	Chr09:46314301..46314552	AAAGGACCTCATGTATAATTC	14
		TTTCCATACTTCGTTACCGAC	454
		ATCTTCTAGCTCCATATTTCC	4
		TGCCATCCAAAGAGTGATTAG	6
		ATTATCCTTTTCCATTTTGGT	9
		GTCCTTTGGGAACACTGCACA	11
		ATATGCAAAACATTGTCTCAA	2
		ATTAAGTGGAAGGTGATGATA	4
		ACTCAGCCTCAGAGCAGGTAG	7
		AATAGAACTTTCATCTTGAGG	10
		ATTATACATGAGGTCCTTTTT	6
		AAATATGGAGCTAGAAGATGT	2
		AATCACTCTTTGGATGGCACA	44
		CAAAATGGAAAAGGATAATCT	18
		TGCAGTGTTCCCAAAGGACAC	3
		TCATCACCTTCCACTTAATTT	10
		ACCTGCTCTGAGGCTGAGTTA	2
		TCAAGATGAAAGTTCTATTCT	38
2	Chr09:46314888..46315139	CATCTAAGACAAGCAAGTATC	2
		GTTTTCCAGTCAATAACTCCT	288

	CCAAGTCCTCAACATCCAGAG	10
	ATCTTCTTTCAATATTTCTTA	3
	CAATTGTCTTTATCAACCTCT	193
	TCTCATCAAAATCATCTGAGA	3
	CACAAACCCATATTTTGGGAT	6
	TGAAATGCTCAGTCACTCTCT	211
	CATCATTGAAGATCATTGTTGG	315
	CAAGTGTCGTCTTTCTAGTC	8
	TACTTGCTTGTCTTAGATGAT	138
	GAGTTATTGACTGGAAAACGA	132
	TCATTTCAGAAGAAGCTTCAG	48
	CTGGATGTTGAGGACTTGGCT	4688
	GGAAATATTGAAAGAAGATCT	4
	AGGTTGATAAAGACAATTGTA	21
	TCAGATGATTTTGATGAGAAG	59
	CCCAAATATGGGTTTGTGTC	24
	AGAGTGACTGAGCATTCAAT	59
	CAAATGATCTTCAATGATGAG	442
	CTAGGAAAGACGACACTTGCC	913

Table 3.5 List of siRNAs generated from *StRGA4 PHAS* locus post *Stu-miR482c* cleavage. Asterisk (*) indicates siRD29(-) and its abundance value (highlighted in yellow).

Position of siRNA	SiRNA sequence	Number of reads	
		<i>Stu-miR482c</i> + <i>StRGA4</i>	<i>StRGA4</i>
D1(+)	ACTAGGAAAGACGACACTTGC	63	29
D2(+)	CCAAATGATCTTCAATGATGA	278	91
D3(+)	GAGAGTGACTGAGCATTTCAA	16	4
D4(+)	TCCCAAATATGGGTTTGTGT	0	0
D5(+)	CTCAGATGATTTTGATGAGAA	0	0
D6(+)	GAGGTTGATAAAGACAATTGT	1	1
D7(+)	AGGAAATATTGAAAGAAGGTC	64	54
D8(+)	TCTGGATGTTGAGGACTTGGC	429	316
D9(+)	TTCATTTCAGAAGAAGCTTCA	0	1
D10(+)	GGAGTTATTGACTGGAAAACG	52	51
D11(+)	ATACTTGCTTGTCTTAGATGA	4	3
D12(+)	TGTTTGGAATGATGATCAAGA	264	206
D13(+)	AAAGTGGGCTAAGTTAAGAGC	698	471
D14(+)	AGTCTTAAAGGTTGGAGCAAG	21	27
D15(+)	AGGTGCTTCTGTTCTAGCAAC	10	10
D16(+)	TACTCGTCTCGAAAAGTTGG	36	25

D17(+)	ATCAATTATGGGAACGTTAGA	70	59
D18(+)	ACCATATCGTTTGTCAAGTTT	47	46
D19(+)	ATCTCAACATGATGGCTTGCT	11	8
D20(+)	GTTGTTTATGCAACGCGCGTT	0	0
D21(+)	TGGGCAACAAAGAGAAATAAA	18	13
D22(+)	TTCTAATCTTGTGGCCGTTGG	77	59
D23(+)	AAAGGAGATTGTGAAGAAATG	82	62
D24(+)	TGGTGGTGTGCCTTTAGCAGC	38	35
D25(+)	CAAGACTCTTGGAGGTATTTT	15	14
D26(+)	ACGCTTCAAGAGAGAAGAAAG	1	0
D27(+)	TGAATGGGAACATGTGAGAGA	182	131
D28(+)	TAATGAGATTTGGAATTTGCC	2724	153
D29(+)	TCAAGATGAAAAGTTCTATTCT	2	0
D30(+)	ACCTGCTCTGAGGCTGAGTTA	6	1
D31(+)	TCATCACCTTCCACTTAATTT	7	6
D32(+)	GAGACAATGTTTTGCATATTG	1	0
D33(+)	TGCAGTGTTCCCAAAGGACAC	0	0
D34(+)	CAAAATGGAAAAGGATAATCT	16	21
D1(-)	AAGTGTCGTCTTTCCTAGTCC	137	80
D2(-)	ATCATTGAAGATCATTTGGGC	46	37
D3(-)	GAAATGCTCAGTCACTCTCTC	9	3
D4(-)	ACAAACCCATATTTTGGGATT	0	0
D5(-)	CTCATCAAAATCATCTGAGAC	0	0
D6(-)	AATTGTCTTTATCAACCTCTT	3	2
D7(-)	CCTTCTTTCAATATTTCTTAC	6	6
D8(-)	CAAGTCCTCAACATCCAGAGA	6	5
D9(-)	AAGCTTCTTCTGAAATGAAGC	0	0
D10(-)	TTTTCCAGTCAATAACTCCTG	101	75
D11(-)	ATCTAAGACAAGCAAGTATCG	8	3
D12(-)	TTGATCATCATTCCAAACATC	9	8
D13(-)	TCTTAACTTAGCCCACTTTTC	21	16
D14(-)	TGCTCCAACCTTTAAGACTGC	65	49
D15(-)	TGCTAGAACAGAAGCACCTCT	124	85
D16(-)	AACCTTTTCGAGACGAGTAGT	714	561
D17(-)	TAACGTTCCCATAAATTGATCC	4	6
D18(-)	ACTTGACAAACGATATGGTTC	2	4
D19(-)	CAAGCCATCATGTTGAGATAA	0	4
D20(-)	CGCGCGTTGCATAAACAACAG	0	0
D21(-)	TATTTCTCTTTGTTGCCCAA	14	9
D22(-)	AACGGCCACAAGATTAGAATT	145	110
D23(-)	TTTCTTCACAATCTCCTTTCC	3	3
D24(-)	TGCTAAAGGCACACCACCACA	16	12
D25(-)	AATACCTCCAAGAGTCTTGGC	5	7
D26(-)	TTCTTCTCTTGAAGCGTAA	0	0
D27(-)	TCTCACATGTTCCCACTTCACT	27	13
D28(-)	CAAATTCCAAATCTCATTATC	2	0
D29(-)*	AATAGAACTTTCATCTTGAGG	43	27
D30(-)	ACTCAGCCTCAGAGCAGGTAG	0	0
D31(-)	ATTAAGTGGAAGGTGATGATA	36	32
D32(-)	ATATGCAAAACATTGTCTCAA	0	0
D33(-)	GTCCTTTGGGAACACTGCACA	3	9
D34(-)	ATTATCCTTTTCCATTTGGT	11	6

Table 3.6 List of 12 tandem TGAC core motifs present within 2.2 kb upstream sequence from TSS of *StGA3ox3* gene.

End (bp)	Start (bp)	Linker (bp)	Pattern (motif orientation)	Matching sequence within 2.2 kb promoter region from TSS
-399	-430	24	TGAC-TGAC	aacc TGAC TCTTTTGAGGGACTTGAAAATATT TGAC gaac
-399	-454	48	TGAC-TGAC	taaa TGAC TGTCTTACAAACCCAAAACCTGACTCTTTTGAGGGACTTGAAAATATT TGAC gaac
-427	-516	82	TGAC-TGAC	gttc TGAC ATGATTTTGAAAAGTTGTGTTTGAACATGCAATATGT TTTTAAAAATTTTGTAAATGACTGTCTTACAAACCCAAAACCT TGAC ttct
-451	-548	90	GTCA-TGAC	tgta GTCA ATATTTGAAACTTGAAAATTTTAAGTTCTGACATGATT TTTGAAAAGTTGTGTTTGAACATGCAATATGTTTTTAAAAATTT TTGTAAAT TGAC tgct
-513	-588	68	TGAC-TGAC	ccct TGAC CCTATCATTTGCTCCGGTTTAATCATAAATTTTGTAGT CAATATTTGAAACTTGAAAATTTTAAGTTCT TGAC atga
-545	-588	36	TGAC-GTCA	ccct TGAC CCTATCATTTGCTCCGGTTTAATCATAAATTTTGTAGT CA atat
-545	-633	81	TGAC-GTCA	gtgt TGAC TTTATATAGATAGTAGTTTTTCGACATCCTCACTCCGC CCTTGACCCTATCATTTGCTCCGGTTTAATCATAAATTTTGTAGT CA atat
-585	-633	41	TGAC-TGAC	gtgt TGAC TTTATATAGATAGTAGTTTTTCGACATCCTCACTCCGC CCT TGAC ccta
-790	-799	2	TGAC-TGAC	aaaa TGAC AA TGAC tagc
-1266	-1292	19	TGAC-GTCA	caca TGAC TAATTATGTCGGGCCCTAC GTCA tcag
-1416	-1462	39	GTCA-TGAC	tata GTCA TATACCTTCAACAATTTATTTCTTCAACGGACTTTTACT TGAC ccat
-1987	-1997	3	GTCA-TGAC	aagt GTCA TCAT TGAC tcga

Part of this work has been published in **Plant Physiology**.

Malankar N, Kondhare KR, Saha K, Mantri M, Banerjee AK (2023) The phased short-interfering RNA siRD29(-) regulates *GIBBERELLIN 3-OXIDASE 3* during stolon-to-tuber transitions in potato. **DOI:** 10.1093/plphys/kiad493.

Chapter 4

Understanding the function of potato-specific miRNA72 in the regulation of tuber formation in potato

4.1 Introduction

Potato tubers are formed as a result of the growth and development of subterranean stems, specifically known as stolons. According to Pasare et al. (2013), the growth of this structure typically occurs from the basal nodes of the stem, resulting in the development of diageotropic branches characterized by elongated internodes. The process of tuber development is regulated by the interaction of key phytohormones, including gibberellin (GA), auxin, cytokinin (CK), and strigolactones (SL), as well as transcription factors, mobile mRNAs, and sucrose signals (Hannapel et al., 2017; Kondhare et al., 2021). Significant advancements have been achieved in understanding the hormonal regulation of this phenomenon. Nevertheless, the mechanisms by which plants regulate the essential hormonal networks involved in tuber development remain unknown.

Small RNAs (sRNAs) have been shown to regulate the hormonal networks in plants, coordinating organ development and stress responses. For example, the regulatory role of the miR394-*LCR* (LEAF CURLING RESPONSIVENESS) pair in the negative feedback loop of CLAVATA-WUSCHEL (CLV-WUS), for shoot apical meristem (SAM) development has been investigated by Knauer et al. (2013). It has also been reported that ARGONAUTE 10 (AGO10) protein sequesters miRNA165/166, thereby leading to the upregulation of HD ZIP III transcription factors (TF), which are essential for the maintenance of the shoot apical meristem (SAM) (Zhu et al., 2011). In a study conducted by Wu et al. (2009), it was shown that miR156, another conserved microRNA, plays a role in regulating the juvenile phase in plants. This regulation is achieved through the repression of miR172, which is known to induce the adult phase. Overexpression of miR156 in potato plants led to the emergence of aerial stolons originating from axillary meristems, which subsequently developed into aerial tubers (Bhogale et al., 2014). Additionally, it was demonstrated that it induces the expression of cytokinin biosynthesis genes, increasing lateral branching. In contrast, miR390 has been shown to target StCDPK1, a calcium-dependent protein kinase, which phosphorylates StPIN4, an auxin efflux carrier. This regulatory mechanism is crucial in modulating auxin transport in tuberizing stolons. According to the stated reports, it is indicated that sRNAs are involved in regulating the process of tuber development. However, their precise role in the transition from stolons to tubers has not been investigated thus far.

The availability of the potato genome, “Genome Sequence and Analysis of the Tuber Crop Potato,” 2011, and the advances in high throughput sequencing technologies have enabled researchers to identify miRNAs, conserved as well as novel that can contribute to the development and stress responses in potato (Xie et al., 2011; Zhang et al., 2013; Lakhotia et al., 2014). Much of the efforts have been undertaken to study the functions of conserved miRNAs across plant species, to understand the biological process, including tuberization in plants. Nevertheless, there is a scarcity of research addressing the function of miRNAs specific to individual species. The gap of knowledge exists when it comes to understanding the tuberization process. Lakhotia et al (2014) attempted to understand the role of miRNAs in tuberization of potato by carrying out small RNA profiling of tuber developmental stages from a day-neutral potato cultivar *Chandramukhi*. Authors identified 89 conserved miRNAs and 147 potato-specific miRNAs that were predicted to be involved in the tuberization process. Using a small RNA sequencing approach, we have also identified a plethora of microRNAs and phasiRNAs (phased secondary siRNAs) that target key tuberization genes in the early stage of tuber development (Kondhare et al., 2018). These unique small RNAs could have the potential to function during stolon-to-tuber transitions of tuber development. Based on the abundance of miRNAs, their putative tuberization-related target genes, and expression patterns during stolon-to-tuber transition stages, we selected putative potato-specific miRNAs, *miR34*, *miR72*, *miR53*, and *miR111* (Lakhotia et al., 2014; Zhang et al., 2013) for study. We validated candidate miRNAs and performed expression analysis in SD induced stolons (tuber inducing conditions) (Fig. 4.1). Interestingly, we noted that, *miR72* significantly reduced under SD conditions suggesting its possible role in tuber development. *Cryptochrome1b* and *Branched 1b/ tuber identity (StBRC1b/StIT1)* genes were identified as putative targets of *miR72*. Recently, *StIT1* has been demonstrated as tuber identity gene in potato (Tang et al., 2022) and shown to interact with the positive regulator of tuberization, *StSP6A*. Hence, we speculate that the regulation of *StIT1* gene via *miR72* might be involved in tuber development in potato.

In this study, to understand the function of *miR72* in tuber development, we have generated *miR72* overexpression lines under a constitutive promoter, which produced belowground sessile tubers indicating that the development of axillary branches from the basal nodes of the stem is affected. Molecular mechanism of the sessile tuber formation and the role of novel *miR72* in this regard is unclear. Such an interesting observation have enabled us to ask the following questions: (1) what are the targets of *miR72* that might have contributed to

the sessile tuber phenotype? (2) How phytohormones are involved in governing stolon formation? (3) How is the expression of miR72 regulated during stolon-to-tuber transitions? And finally, (4) Does miR72-*StIT1* (gene-target pair) regulate sessile tuber development phenomenon?

4.2 Material and methods

4.2.1 Plant growth and conditions

In this study, a photoperiod-responsive potato cultivar—*Solanum tuberosum* ssp. *andigena* 7540, which tuberizes under short-day conditions, and a day-neutral cultivar *Solanum tuberosum* L. cv. Désirée was used. Wild-type (WT) plants were propagated by sub-culturing nodal explants on Murashige and Skoog's (MS) media supplemented with 2% sucrose (w/v) (Murashige & Skoog, 1962). *In vitro* potato plants were maintained in a plant growth incubator (Percival Scientific) at 24 °C under long-day (LD; 16 h light/8 h dark) conditions. Post four weeks, Désirée plants were transferred to soil and maintained at 22°C under LD conditions in the greenhouse until maturity. To score tuber phenotype whereas andigena plants were grown for 10 weeks under LD conditions until they attained 10-12 leaves stage. Subsequently, half of the plants were transferred to the tuber-inducing SD conditions, and the remaining plants were maintained under LD photoperiod for expression analysis.

4.2.2 Validation of miRNA72 in potato

MiRNA72 (miR72) sequence was provided as a query for BLAST search in PGSC database (<http://spuddb.uga.edu/>), the resulting matched region with 100bp upstream and downstream sequences were retrieved and used as the precursor of miR72. MFold (Zuker, 2003) was used to predict the secondary structure of premiR72. The selected sequence was then amplified from total RNA harvested from leaves of WT andigena plants. cDNA was prepared with oligo (dT) primers, and was followed by a PCR for premiR72. The mature form of miR72 was detected using stem-loop RT-PCR as described (Varkonyi-Gasic et al., 2007). In brief, total RNA was isolated from SD-induced stolon samples using RNAiso Plus (DSS TAKARA) as per the manufacturer's protocol. One µg of total RNA was reverse-transcribed using stem-loop primers (Table 4.1) to amplify the mature form of the miR72. End-point PCR was performed with miR72FP and universal reverse primers. PCR amplified product further sequence confirmed. Primer details are given in the table (Table 4.1).

4.2.3 Bioinformatic prediction and cleavage site mapping analysis for miR72 targets

Candidate targets of miRNA72 were predicted with the help of psRNATarget tool (plantgrn.noble.org/psRNATarget (Dai et al., 2018)). Default parameters were used with an expectancy-value ≤ 3 to avoid false targets. Interesting candidates with lower expectancy values (Table 4.2) were considered for further analysis. To validate targets of miR72, 5' RLM-RACE assay was performed using the First Choice RLM-RACE kit (Ambion). Briefly, total RNA was extracted from stolons from wild-type potato (andigena) plants by TRIzol reagent and ligated to the 5' RNA adapter. cDNA synthesis was performed using oligo (dT) primers. Two sets of PCRs were performed with adapter-specific forward primers and gene-specific reverse primers (Table 4.1). PCR amplicons from secondary-nested PCRs were then cloned in a sub-cloning vector pGEM-T-easy, and sequence verified to identify the cleavage sites of miR72 on target gene transcripts.

4.2.4 Expression analysis of miR72

For expression analysis, four tissue types (shoot-apex, leaf, node and stem) were harvested from two week old soil-grown andigena plants. To investigate the effect of SD photoperiod, tissue types along with samples comprising stolon-to-tuber transition stages (stolon, swollen stolon, and mini-tuber) were harvested at 10th days after their transfer to SD. Leaves were also harvested on the earlier time-points (4th and 7th day after SD induction). Total RNA was isolated from 100 mg of crushed tissues using RNAiso Plus (DSS TAKARA) as per the manufacturer's protocol. miRNA-specific complementary DNA (cDNA) synthesis was carried out using 2 µg of total RNA, Superscript IV Reverse Transcriptase (SSIV-RT Invitrogen), and miRNA-specific stem-loop primers (Varkonyi-Gasic et al., 2007). Quantitative PCR reactions were performed on a CFX96 Real-Time System (Bio-Rad) using sRNA-specific forward and universal reverse primers (Table 4.1) Reactions were prepared with SYBR green master mix (TAKARA Clontech), and incubated at 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s, gene-specific annealing temperature for 10 s, and extension at 72 °C for 10 s. PCR specificity was checked by melting curve analysis and data were analysed using $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.2.5 Gene construct designs and transgenic lines generation

For constitutive overexpression (OE) gene constructs, the precursor sequence of miRNA72 (126 bp) was amplified by reverse transcription PCR from the leaves of soil-grown andigena plants using primers listed in Table 4.1. Further the sequence was cloned into the

binary vector, pBI121 downstream to a constitutive CaMV35S promoter. For the generation of knockdown lines, Short Tandem Target Mimic (STTM) approach (Tang et al., 2012) was undertaken. STTM72 construct was designed according to the previous protocol (Tang et al., 2012) with a few modifications. Two primers with the sequence that can form hairpin were synthesized based on a sequence of mature miR72 (Table 4.1). 48nt linker sequence that can form hairpin was used. The *tcg* bulge site was included in the middle of miR72 complementary sequences. Primers were annealed to a sub-cloning vector pGEM-T-easy and PCR amplified. Resultant product was circularized and STTM sequence (94 bp) was removed via restriction digestion. Subsequently STTM sequence mobilized into pCB201 binary vector downstream to a constitutive CaMV35S promoter. Both constructs were then transformed into *Agrobacterium tumefaciens* strain GV3101. Transgenic potato plants were generated using the *Agrobacterium*-mediated leaf transformation method (Banerjee et al., 2006). The transgenic line of *andigena* harboring 35S::*GUS* construct was considered as a vector control (VC). Phenotypic characteristics, such as plant growth, leaf shape and leaflet numbers in transgenic lines were recorded before the initiation of SD photoperiod, whereas the tuber and root biomass were measured after four weeks of SD induction. Prior to transgenic lines generation, 35S::*PremiR72*-pBI121 construct harboring *Agrobacterium* was infiltrated into the *N. benthamiana* leaves and miR72 was detected in the infiltrated leaves by stem-loop PCR.

4.2.6 Northern blot for the detection of miR72

Northern blot was carried out for small RNAs as described in the previous protocol (Tirumalai et al., 2020) with minor modifications. Briefly, a total of 20-25 ug of RNA was re-suspended in RNA loading buffer equal volume (not more than 10 ul) of deionized formamide (Merck) heated at 98°C for 2mins and loaded on 15% polyacrylamide denaturing gel (19:1 ratio for acrylamide to bis-acrylamide, 8M urea). The gel was run at 60V for 3-4 hours and RNA from the gel was transferred to hybond N⁺ membrane (Ameshram) by electroblot transfer in 1X tris borate EDTA buffer (TBE) at 10V for overnight. RNA was UV-cross-linked to the membrane. The hybridization was performed at 35 °C for 24 hrs in UltraHyb-oligo buffer (Ambion) with the oligo probes (Table 4.1) end labeled with ³²P by a poly-nucleotide kinase (NEB). The blots were washed with 2X SSC buffer + 0.1% SDS twice for 15 min at 35 °C. Final wash performed with 0.1X SSC buffer at same conditions. Further, blots were kept in a phosphor screen, exposed for 5 days and the signal was captured using Typhoon phosphorimager (GE healthcare).

4.2.7 Primer List

The primer details of the primers used in this study are listed in Table 4.1.

Table 4.1. List of primers used in this study.

Sr. No	Primer name	Primer Sequence (5'→3')
1	StmiR72-STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACCTTAGA
2	StU6-STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACTTGGAC
3	miR34STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACCTTCCA
4	miR53STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACACCCAA
5	miR111STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACTCAAAT
6	miR72FP	GCGGCGTTGGTTGAGTGAGCA
7	U6FP	GACACGCACAAATCGAGAAATG
8	miR34FP	GCGGCGTCTGTGACAGGATAA
9	miR53FP	GCGGCGTTCCATGAGACTGTTT
10	miR111FP	GCGGCGAACATCTCCAGCCAT
11	Univ-RP	AGTGCAGGGTCCGAGGT
12	premiR72-BamHI-FP	GAGAGGATCCGTTGATTTCTCGGGTGCTTACTCAAC
13	premiR72-SacI-RP	GAGAGAGCTCGTCGATTTCTTAGATGCTCACTCAACC
14	Elf3e qFP	GGAGCACAGGAGAAGATGAAGGAG
15	Elf3e qRP	CGTTGGTGAATGCGGCAGTAGG
16	StCRY1b-FP	CAGTTGTGGATAGTAGAGCAGA
17	StCRY1b-RPo	AACGAGACTTCAATCCCTTTCGAC
18	StCRY1b-RPi	CCAGTACTTATGGGTAAGAAGTGCAG

4.2.8 Accession List

Table 4.2 The accession numbers for the transcripts analyzed in this chapter:

Sr. No.	Gene Name	Accession	Source
1	<i>StBRC1b</i>	Soltu.DM.06G025210.1	PGSC
2	<i>StCRY1b</i>	Soltu.DM.12G007030.1	PGSC
3	Polcalcin-jun-o	Soltu.DM.02G034080.1	PGSC
4	Cold inducible histidine kinase	Soltu.DM.02G023620.1	PGSC

4.3 Results

4.3.1 Validation of potato-specific miRNA72 in potato

Previous small RNA sequencing studies (Lakhotia et al., 2014; Ou et al., 2015) have reported potato-specific miRNAs in leaves and stolons. The authors reported that miR72 have an abundant expression in leaves and tubers. Further, our deep sequencing analysis also confirmed its presence in andigena cultivar. In our analysis, 126 bp of premiR72 was found to be present on chromosome 12 with genomic locus (Chr12::3975263, 3975388), in potato. We validated the presence of miR72 and its precursor in photoperiod sensitive variety andigena and a photoperiod neutral potato variety Desiree. A 21 bp mature miR72 was detected by stem-loop end point PCR from leaves and stolons for both varieties (Fig. 4.2 A). To confirm the presence of premiR72, we performed RT-PCR from leaves of both potato varieties (Fig. 4.2 B), followed by a PCR amplification, which were sequence confirmed. Further, we found that the precursor of miR72 was found to be located in the first intron of an actin-binding protein, profilin (Soltu.DM.12G025540) (Fig. 4.3 A). Through MFold, we predicted structure (Zuker, 2003) of premiR72 and found that it had a typical hairpin loop structure with miR72 sequence present in the stem region, which is a characteristic of miRNA precursors (Fig. 4.3 B). Overall, these results demonstrated that miR72 is expressed in both potato cultivars, andigena and Desiree.

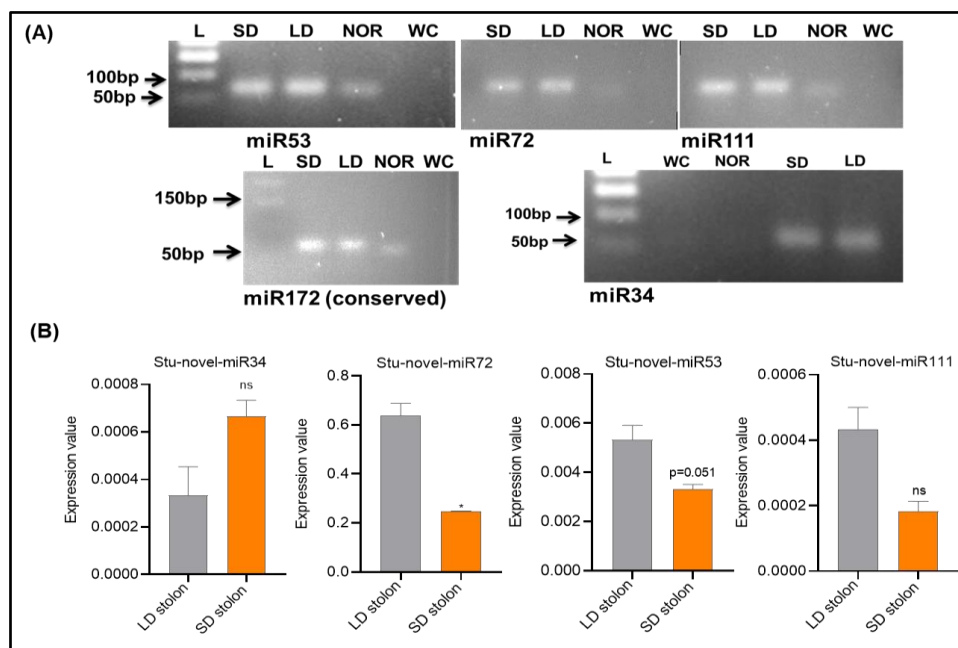


Fig. 4.1 Detection of potato-specific miRNAs and their expression analysis in stolons under photoperiodic conditions. (A) A 3% agarose gel electrophoresis image for the amplification of *miR53*, *miR72*, *miR111*, and *miR34* from SD-induced leaves from potato plants using stem-loop RT PCR. L=50 bp ladder, NOR= stem-loop primer control, WC= water control. Detection of Conserved *miR172* is served as positive control. (B) Expression levels of candidate miRNAs in stolons from LD and SD induced andigena plants (14 days). *StU6* was used as the reference gene. Error bars indicate standard error of means (SEM) from three biological replicates (n=3). Asterisks indicate significant differences at $p < 0.05$, using paired student's t-test. ns = not significant at $p < 0.05$.

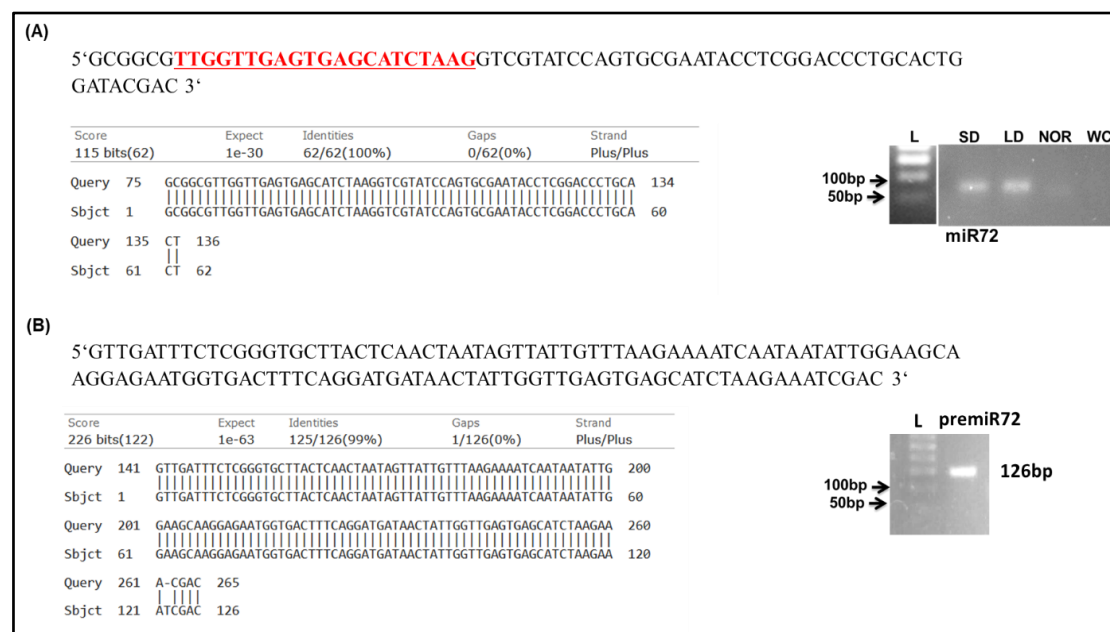


Fig. 4.2 Detection of miR72 and its precursor from short day (SD) induced leaves and their confirmation by Sanger's sequencing. A 3% agarose gel electrophoresis image for amplification of *miR72* (A) and precursor *miRNA72* (B) from SD-induced leaves using stem-loop RT-PCR followed by PCR respectively. L=50 bp ladder, NOR= stem-loop primer control, WC= water control. Stem-loop PCR amplified product (A) and premiR72 amplicon (B) were cloned into a sub-cloning vector pGEM-T-easy and the sequence was confirmed using Sanger sequencing for multiple clones. The resulting representative sequences are shown.

4.3.2 Abundant expression of miR72 in different tissue types of potato

The relative expression of *miR72* and its precursor were analyzed in different potato tissue types with quantitative RT-PCR. *miR72* was ubiquitously expressed in all tissue types, with the highest expression in leaves and least expression in shoot apex (Fig. 4.3 C). It was observed that *miR72* levels were altered in the SD-induced stolons, which further decreased during the stolon-to-tuber transition (Fig. 4.3 D). This altered expression of *miR72*, suggests a possible regulation of *miR72* in tuber developmental stages. Further, the level of *miR72*

precursor decreases in stolon-to-tuber transition stages (Fig. 4.3 E) similar to miR72, indicating an upstream regulation of premiR72 might contribute to the reduced miR72 expression.

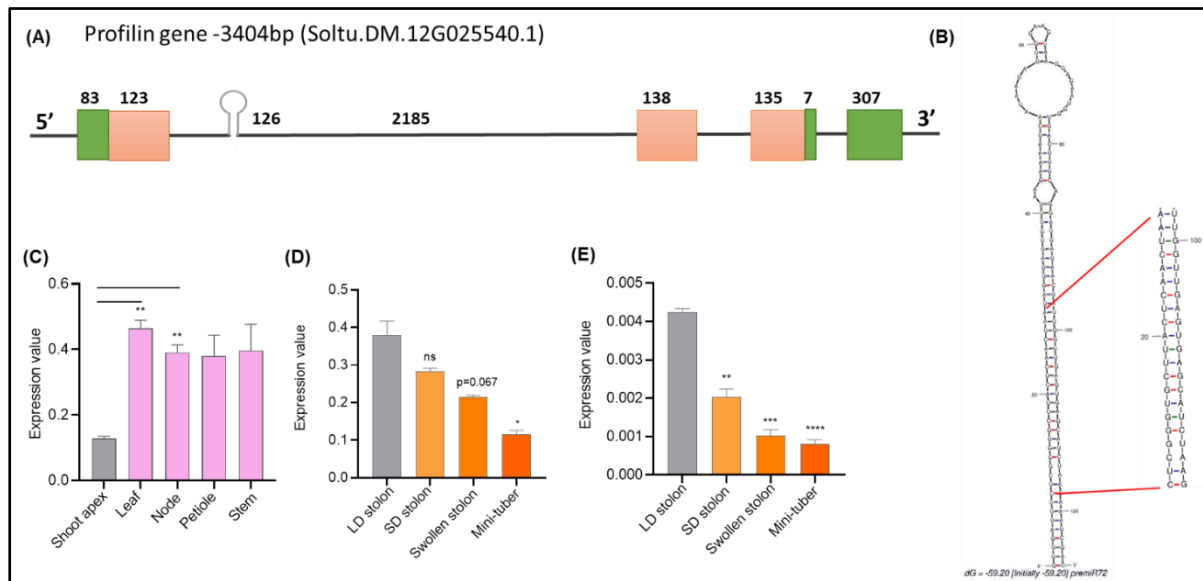


Fig. 4.3 Intronic miR72 exhibits tissue-specific expression pattern and reduces during stolon developmental stages. (A) A schematic representation of genomic locus of profilin gene. Precursor miR72 is located within first intron (2185 bp) of the gene. Exons are highlighted in orange boxes whereas UTRs are represented by green boxes. Lengths of the region are indicated by numbers at the top. (B) Secondary structure of premiR72, as predicted by MFold (Zuker, 2003). MiR72 sequence is further brought into the focus. (C) Expression of miR72 from the two-week old potato plant in different tissue types. *StU6* was used as the reference gene to normalize the expression of miR72. The abundance of miR72 was plotted using $2^{-(\Delta Ct)}$ method (Livak and Schmittgen, 2001). The levels of miR72 (D) and precursor of miR72 (E) are represented during stolon-to-tuber transition stages. Error bars indicate standard error of means (SEM) from three biological replicates (n=3). Asterisks (one, two, three and four) indicate significant differences at $p < 0.05$, $p < 0.01$, $p < 0.005$, and $p < 0.001$ respectively, using one-way ANOVA. ns = not significant at $p < 0.05$.

4.3.3 Overexpression (OE) of miR72 affects tuber and root development, and leads to sessile tuber formation in potato

To characterize miR72 function in potato, six constitutive *35S::premiR72* lines in andigena were generated and two were selected for further studies (Fig. 4.4 A-B). Of all the transgenic lines generated the transgenic line labelled, #OE32 exhibited severe changes in leaf shape and increased trichome numbers, compared to WT (Fig. 4.4 B-D). However, other lines did not exhibit a similar phenotype (Fig. 4.4 B-D). The OE line, #OE30, is shown as a representative line. Further to reconfirm this phenotype was due to miR72, six constitutive OE

lines in andigena were again generated. Based on the expression, three independent lines (referred to as #OE2, #OE4, #OE5) with moderate to high levels of *miR72* expression were chosen for further analysis (Fig. 4.5A-B). The OE lines, #OE1 and 4 did not show any phenotypic effect on plant architecture but, lines #OE2, 3, and 5 exhibited moderate phenotype for increased trichome density and leaflet curling in accordance with #OE32 (Fig. 4.5 C).

Tuberization potential of overexpression lines was evaluated. #OE2, #OE4, #OE5 and #OE32 lines exhibited a severe reduction in tuber weight compared to WT (Fig. 4.6A-C, G). Interestingly, tuber numbers per plant did not change (Fig. 4.6 D, H), but root biomass in these OE transgenic lines was greatly reduced (Fig. 4.6 F, J). In addition, we found that selected *miR72* overexpression lines displayed belowground sessile tubers (Fig. 4.7 A-B). We suspected the phenotype might be photoperiod dependent. To confirm this hypothesis, we generated *miR72* overexpression lines in a day-neutral cultivar Desiree. We observed that the OE transgenic lines generated in Desiree also produced sessile tuber phenotype (Fig. 4.7 C-D).

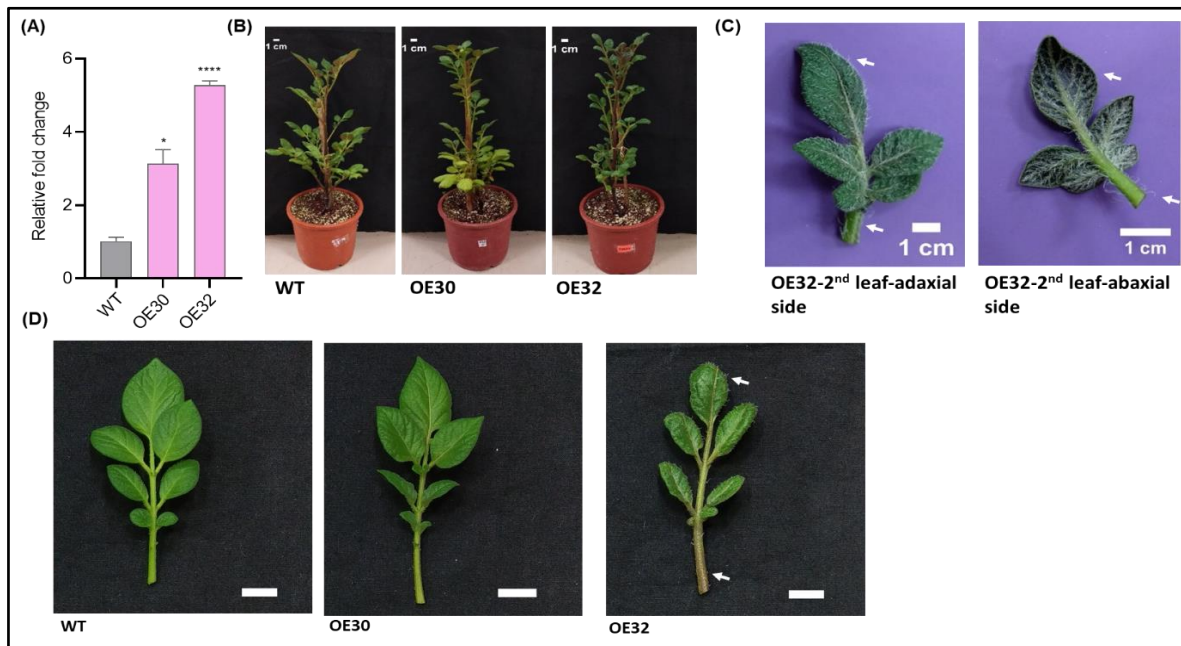


Fig. 4.4 MiRNA72 overexpression affects leaf architecture in transgenic lines in Desiree. (A) Expression levels of *miR72* in overexpression (OE30 and 72OE32) lines compared to WT. *StU6* was used as a reference gene for stem-loop quantitative RT-PCR analysis. Error bars indicate standard error of means (SEM) from three biological replicates (n=3). Asterisks (one and four) indicate significant differences at $p < 0.05$ and $p < 0.001$, respectively using Student's t-test. (B) Plant architecture of *miR72*-OE lines along with WT. Scale bar= 1 cm. (C) Increased trichome number on young leaf (second leaf from shoot apex) of OE32 line is shown. Arrow indicates the trichomes. (D) Representative fully

expanded (5th) leaf images from *miR72*-OE lines and WT. The arrow indicates the change in leaflet shape and increased trichome density as compared to WT. Scale bar= 1cm.

To assess the effect of *miR72* knockdown, we also generated STTM lines of *miR72* in *andigena*. We selected three independent lines (Referred as #KD9, #KD10, and #KD25) with more than 80% reduction in *miR72* levels (Fig. 4.8A-B). STTM lines did not have any effect on plant architecture. But, Line #KD9 exhibited tubers with a size bigger than WT plants. However, phenotypic assessment needs to be repeated in order to conclude the effect of *miR72* knockdown in potato plants.

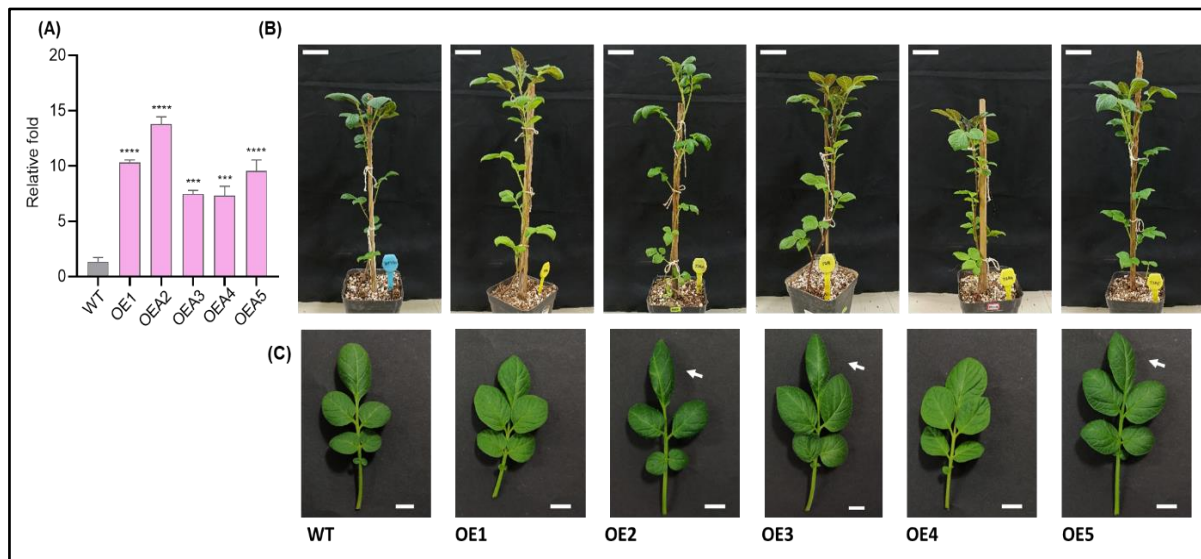


Fig. 4.5 *MiRNA72* overexpression showed moderate phenotype for leaf architecture in transgenic lines. (A) *MiR72* levels in five overexpression lines compared to WT. *StU6* was used as a reference gene for stem-loop in quantitative RTPCR analysis. Error bars indicate standard error of means (SEM) from three biological replicates (n=3). Asterisks (three and four) indicate significant differences at $p < 0.005$ and $p < 0.001$, respectively using Student's t-test. (B) Plant architecture of *miR72*-OE lines along with WT. Scale bar= 5 cm. (C) Representative images for fully expanded (5th) leaf from *miR72*-OE lines and WT. The arrow indicates change in leaflet shape as compared to WT. Also, lines 72A2, 72A3, and 72A5 had moderate increased trichome density with respect to WT. Scale bar= 1cm.

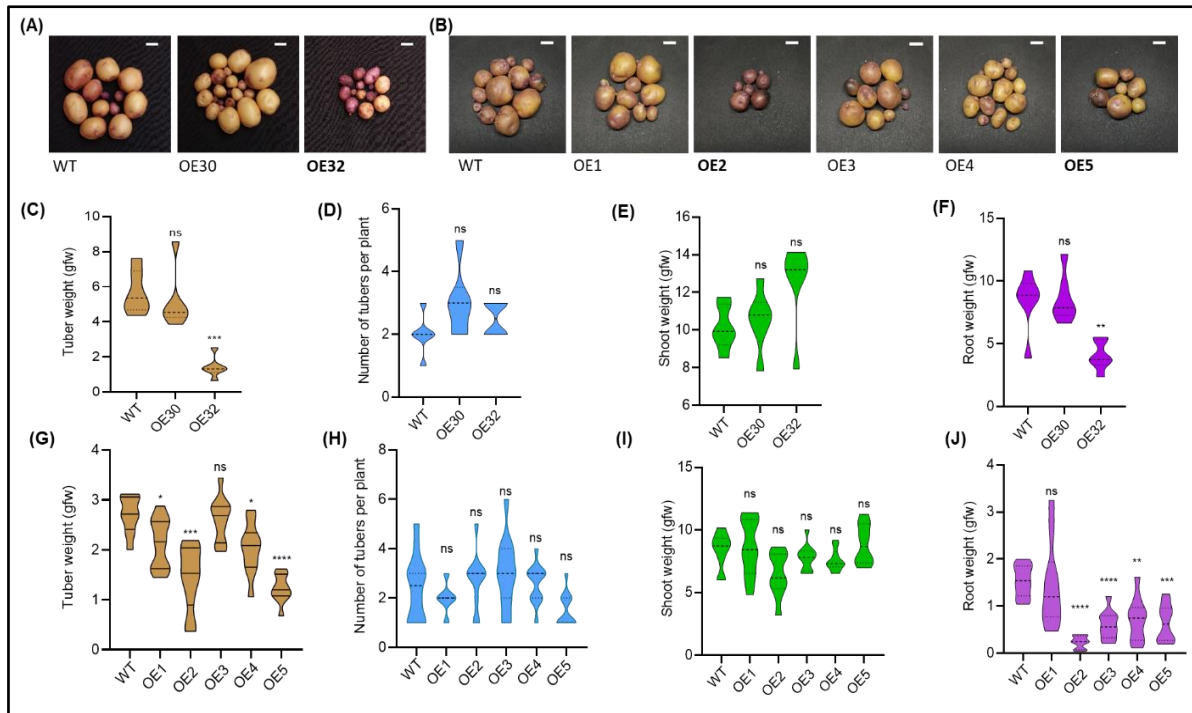


Fig. 4.6 Overexpression of miRNA72 affects tuber and root development in transgenic lines. (A) And (B) Representative images for the tubers were obtained. Each image contains tubers from 6 plants per line. Scale bar= 1 cm. Highlighted lines (#OE32, #OE2 and #OE5) exhibited significant reduction in tuber weight. Total tuber weight (C) and (G) as well as tuber numbers (D) and (H) are represented in the form of violin plots. Total Shoot weight (E) and (I) is indicated. Reduced root weight (F) and (J) in miR72 overexpression lines is presented. Data from 6 independent plants for each line (n=6) is represented. Asterisks (one, two, three and four) indicate significant differences at $p < 0.05$, $p < 0.01$, $p < 0.005$ and $p < 0.001$, respectively using one way ANOVA test.

4.3.4 miR72 targets interesting candidate genes in tuberization

To explore the cause of phenotypes, observed miR72 overexpression lines of potato, *in silico* prediction of miR72 targets was carried out (Table 4.2). Interesting candidate genes were predicted as a target of miR72 that included Cold inducible histidine kinase, Polcalcine jun-o, a TCP transcription factor (StBRANCHED1b / StIT1) and Cryptochrome1b. A 5' RLM-RACE was performed to identify cleavage site of miR72 on these targets. Only, cryptochrome 1b (*StCRY1b*) was validated as a target with a cleavage frequency (8/8) (Fig. 4.8 C). We could not confirm *StBRC1b* by cleavage site mapping assay however, its expression was decreased (Fig. 4.8 D) in leaves of miR72 OE line (#OE4). Future experiments are being conducted to unravel the role of miR72 in tuber development.



Fig. 4.7 miR72-OE lines exhibited belowground sessile tuber formation. (A) Representative images for sessile tubers observed in the lines 72A3, 72A4 and 72A5. Scale bar= 1 cm. (B) Zoomed image for sessile tubers in 72A5 line. (C) Representative plant and sessile tuber images for miR72-OE desiree transgenic lines (72D3 and 72D4). (D) Zoomed image of sessile tubers in 72D4 line.

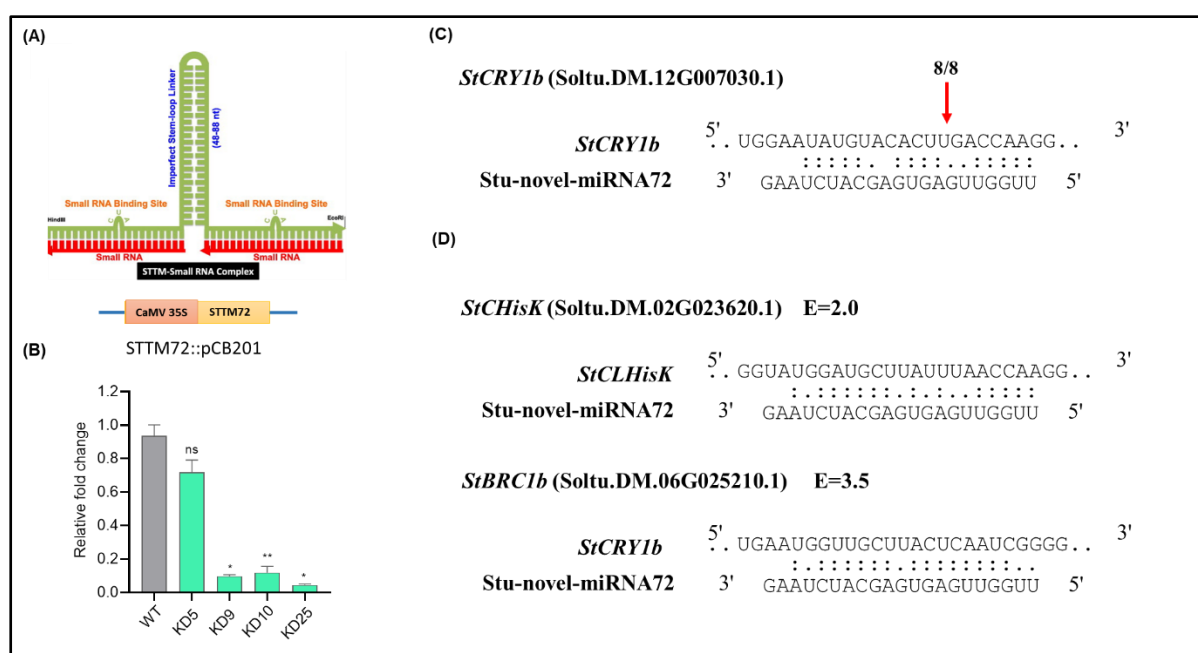


Fig. 4.8 Knockdown lines generation using STTM approach and cleavage site mapping assay for miR72 targets. (A) Schematic image for STTM RNA structure and the putative complex formed

between STTM and the small RNAs. Note: The STTM linker (48nt) forms a weak imperfect “stem-loop” that provides stability to STTM transcript for effective function. (B) MiR72 levels in three knockdown lines compared to WT. *StU6* was used as a reference gene for stem-loop RT-qPCR analysis. Error bars indicate standard error of means (SEM) from three biological replicates (n=3). Asterisks (one and two) indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively using Student’s t-test. (C) A modified 5’ RLM-RACE results representing the cleavage site mapping of miR72 on *StCRY1b* transcript. The numbers (8/8) represent the frequency of clones showing the same cleavage site. (D) Cold inducible histidine kinase (*StCHisK*) and TCP transcription factor/tuber identity gene (*StBRC1b*) Putative targets of miR72 as predicted by psRNATargetfinder software with expectancy less than 3.0.

4.4 Discussion

Small RNA (sRNA) mediated hormonal networks regulate various plant developmental processes, such as SAM initiation and its maintenance, leaf polarity establishment and root development (Dong et al., 2022). A stolon becomes the focal point wherein, all the hormone regulated gene networks co-ordinate a series of developmental events to form mature tuber. We speculate that small RNAs might play a key role in governing tuberization. We have earlier reported that a number of sRNA candidates could potentially be involved in early stages of tuber development (Kondhare et al., 2018, 2021). Here, we have attempted to explore the putative function of a potato specific miRNA, miR72, during sessile tuber development in potato.

4.4.1 miR72 expression and its possible regulation during stolon-to-tuber transitions.

MiR72 exhibited the highest reads amongst potato specific miRNA population from two different small RNA sequencing datasets (Zhang et al., 2013; Lakhotia et al., 2014; Ou et al., 2015). Moreover, it is found to be abundant in aerial tissue types, specifically in leaves (Fig. 4.3 C). miR72 levels were reduced across the stolon-to-tuber transition stages (Fig. 4.3 D-E), which matches with the expression of miR72 precursor, suggesting that both might be regulated during the tuber development. However, we observed that miR72 is located within the first longest intron of a potato profilin gene. Profilin is an actin monomer –binding protein, ubiquitously expressed in eukaryotes (Meagher and Williamson., 1994). Actin is a major constituent protein of microfilaments in eukaryotic cells justifying the higher abundance of miR72 in potato tissue types (Fig. 4.3 C). We did not observe any change in profilin levels in stolon stages indicating that the reduction observed in miR72 levels in stolons, is independent of the parent gene expression. The decreased expression of precursor in accordance with miR72, suggests that processing of miR72 from its precursor was not hampered. Therefore, we

speculate the possibility of an alternative splicing event (AS) within the intronic region of profilin gene. In eukaryotes, AS has been found to be an important regulatory mechanism in different cell types and at different developmental stages, as well as in response to environmental cues (Yang et al., 2012; Yang et al., 2012). In a study by Yan et al (2012), heat stress are shown to trigger alternate splicing event (AS) to suppress intronic miR400 expression. Previous study (Ou et al., 2015) reported that novel miR72 reads are reduced under cold stress in potato tubers. We also observed that miR72 expression was reduced post cold stress, indicating that miR72 expression can be regulated by abiotic stress. Subsequently, through an *in silico* approach, we have identified five AS sites within the intronic region of profilin gene. Further investigations are required to confirm alternate splicing as a regulatory mechanism for miR72 reduction during the tuber development.

4.4.2 Altered expression of miR72 affects tuber and root development.

To understand the role of miR72 in tuber development, we had generated transgenic lines for miR72. We observed that *miR72-OE* lines exhibited impaired leaf development as well as reduced tuber yield and root biomass. To confirm the reproducibility of the results, we generated *miR72-OE* transgenic lines two to three times. Out of five positive lines tested for tuber development, only line #OE32 exhibited extreme phenotypes such as increased trichome density, impaired leaf development and reduced tuber and root biomass. In the second set of plant transformation events, we obtained three lines (#OE2, #OE4 and #OE5) with moderate trichome density and leaf curling phenotype whereas, strong reduction in tubers and root yield suggests that constitutive expression of miR72 may be the cause for these phenotypes.

StBRC1b/StIT1, the miR72 target is a TCP transcription factor (TFs). TCP TFs have been shown to regulate leaf and root development processes, branching, lateral organ development, circadian rhythm and gametophyte development (Li, 2015) via stimulating brassinosteroid, jasmonic acid and flavonoid biosynthesis as well as auxin signaling genes. In a recent study by Nicolas et al (2022), *StBRC1bi* potato lines exhibited ectopic aerial tuber formation and reduced below ground tuberization. These lines had defect in leaf shape compared to WT. In our study, we did not observe any aerial tubers but, other phenotypes were quite noticeable. It was also observed that only #OE4 line exhibited ~40% decrease in *StBRC1b* gene expression. As miR72 expressed abundantly in all tissue types, we think for a possible tissue specific regulation of target genes by miR72. To confirm this, currently we are performing the tissue specific expression analysis of target candidate genes in the transgenic

lines. Further, we had generated *miR72-KD* lines in potato *via* STTM approach. We observed a ~85% reduction in the miR72 levels with no phenotypic alterations in leaves of KD lines. These lines exhibited larger potato tubers compared to WT. However, our findings are preliminary at this point because, the phenotypic analysis needs to be repeated to obtain conclusive results. As miRNA72 has several putative targets, we did not rule out the possibility that phenotype can be due to another target candidate gene. We confirmed Cryptochrome1b as miR72 target with cleavage site mapping assay. However, we could not get any conclusive results for other targets probably due to tissue specific targeting of genes. Additional experimental evidences are required to support this notion.

4.4.3 Constitutive overexpression of miR72 induces belowground sessile tuber formation

MiR72-OE lines exhibited an interesting sessile tuber phenotype suggesting that miR72 might be involved in stolon development. To re-validate, we generated *miR72-OE* lines in Desiree background (a day neutral cultivar) and observed two out of the four lines showing sessile tuber phenotypes. As reported in the study (Nicolas et al., 2022; Tang et al., 2022), none of the *StBRC1b* RNAi lines showed visible sessile tuber phenotype. Tang et al., 2022, shown that *it1* lines lost tuber forming capacity. We did not find any such phenotype in OE lines. Moreover, *miR72-KD* lines did not exhibit sessile tuber formation. All of the above results point towards that sessile tuber phenotype can be an effect of other miR72-target gene/s. Alternatively, a crosstalk of phytohormones could be involved in sessile tuber formation. For e.g. strigolactone (SLs) biosynthetic mutant (*CCD8i*) in potato (Pasare et al., 2013) exhibited a reduced diageotropic growth of the stolons. TCP TFs in Arabidopsis have shown to affect SLs signaling (Minakuchi et al., 2010). Localized down-regulation of bio-active GAs leads to reduction in stolon length to form tubers with reduced stock (Bou-Torrent et al., 2011; Roumeliotis et al., 2013). Interestingly, Luisi et al (2011) proposed that GAs could change bud sensitivity to application of synthetic SLs and SLs induces inhibition of tuber sprout growth. Overall, our preliminary findings suggest that sessile tuber formation is an indeed effect of miR72 overexpression and it can be due to the impaired stolon development. To identify whether this effect is direct through miR72 target or indirectly by the modulation of hormonal network need to be further investigated.

Overall, in this study, we have attempted to identify the function of a highly abundant potato specific miRNA (miR72) during tuber development. MiR72 expression decreases during stolon-to-tuber developmental stages. Overexpression lines of miR72 exhibited reduced

tuber and root biomass, suggesting that reduced level of miR72 is necessary for tuber formation. Moreover, *miR72-OE* lines showed sessile tuber formation. Candidate miRNA-target gene responsible for sessile tuber phenotype has not been investigated yet. We speculate that this phenotype might be due to direct miRNA72 target or indirectly by regulating hormonal signaling pathway. We also observed that miR72 expression might be regulated through AS events. Experiments are underway to show the miR72 function in regulating tuber development.

Table 4.3. *In silico* target prediction for miR72 in potato:

Sr. No	Predicted targets (PGSC accession number)	Score	Mode of Action
1	Cryptochrome 1b, Soltu.DM.12G007030.1	3.0	Cleavage
2	TCP Transcription factor, Soltu.DM.06G025210.1	2.5	Cleavage
3	Cold inducible histidine kinase, Soltu.DM.02G023620.1	2.0	Cleavage
4	Polcalcine jun-o Soltu.DM.02G034080.1	2.5	Cleavage
5	GTP binding 2, Soltu.DM.02G033590.1	3.0	Cleavage
6	Dip2/Utp12 Family domain containing protein Soltu.DM.03G030470.1	2.5	Cleavage
7	Multidrug resistance-associated protein 2, 6 (Mrp2, 6), abc-transporter, Soltu.DM.01G023300.1	2.5	Cleavage
8	Carbohydrate transporter, Soltu.DM.05G021970.1	3.0	Translation
9	PHB2, Soltu.DM.10G003950.1	3.0	Cleavage
10	mRNA splicing factor, Soltu.DM.02G012090.1	3.5	Cleavage
11	E3 ubiquitin-protein ligase UPL5 Soltu.DM.05G024460.1	3	Cleavage
12	ATP-binding cassette transporter Soltu.DM.08G016370.1	3	Cleavage

Summary and future directions

Tuber development (tuberization) in potato is a complex process, which is under the tight control of environmental and internal gene regulatory factors (Hannapel et al., 2017; Kondhare et al., 2020). Numerous studies have focused on identifying factors involved in tuberization. However, only a few reports demonstrate the how actual events of stolon-to-tuber as an organ development happens (Roumeliotis et al., 2012). At the onset of tuberization, a sub-axillary branch of the main stem acquires a series of changes at its tip where the orientation of cell division from sub-apical region changes from transverse to longitudinal, resulting in a radial expansion of stolon, to form a swollen stolon, which accumulates starch to develop into a mature tuber. Phytohormones, such as gibberellin, auxin, cytokinin, abscisic acid and strigolactones have been demonstrated to regulate tuber development. Particularly, a reduction in gibberellin (GA) and a peak in auxins levels are possibly key events governing the stolon-to-tuber developmental transitions. Previous studies have demonstrated the regulation of GA:auxin ratio in stolons by controlling the expression of their metabolism genes (Kloosterman et al., 2007; Carrera et al., 2000; Bou-torrent et al., 2011; Roumeliotis et al., 2013). But how do plants maintain the hormonal balance in stolons remains unclear. Moreover, events such as induction of other phytohormones (cytokinin and jasmonic acid), initiation of biochemical pathways of secondary metabolites and the changes in metabolic flux takes place during the tuber formation process. We speculate that these events are tightly regulated during the tuber development process.

Small non-coding RNAs play a central role in regulating various aspects of plant growth and development. Based on the functionality of small RNAs in organ development in other plants, we hypothesized that small RNAs could govern hormonal networks during stolon-to-tuber transition in potato. Previous reports have identified miRNA populations from the leaves and tubers of potato plant (Yang et al., 2009; Zhang et al., 2009; Xie et al., 2011; Zhang et al., 2013). Lakhota et al. (2014) used a day neutral potato cultivar for miRNA identifications; therefore, the effect of photoperiod was not observed on miRNA expression patterns. As tuberization is greatly affected by the day length (Ewing and Struik., 1992; Rodriguez-Falcon et al., 2006), we hypothesize that photoperiod might have an effect on small RNA expressions in stolon stages. To understand the regulation of stolon transitions under differential photoperiodic conditions, we performed a small RNA sequencing in stolons at different time points of stolon-to-tuber transitions under SD and LD conditions. We identified several candidate microRNAs with putative roles in tuber development. From these datasets, we also identified numerous phasiRNA producing loci that could generate phased siRNAs targeting

key tuberization genes. Further, we selected one such phasiRNA module that generates a novel phasiRNA targeting a GA biosynthesis gene and demonstrated its function in maintaining GA levels during stolon-to-tuber formation. We also attempted to study the function of potato specific miRNA in tuber development. To investigate the functions of small non-coding RNAs during stolon-to-tuber transition, we focused our study on three major objectives.

1. To identify small RNAs from early tuber transition stages of potato under LD vs SD differential photoperiod conditions.
2. To decipher the role of a novel phasiRNA [siRD29(-)] in stolon-to-tuber transition stages.
3. Investigating the function of a potato-specific miRNA (miRNA72) during potato development.

Chapter 1: Introduction

In this chapter, we have conducted a thorough literature survey on the tuberization pathway and small non-coding RNAs regulating plant organ development and stress responses. We have also described the recent advances in the tuber development process. Phytohormonal crosstalk during the stolon-to-tuber transition event is also elaborated. This information suggested the necessity of internal regulatory factors to govern this transition. As small RNAs can orchestrate hormonal networks during plant organ development, we hypothesize that tuber transitions might be under the control of small RNAs. This chapter further describes miRNAs and phasiRNAs as subsets of small RNAs, their biogenesis and the mode of action. We have outlined many examples of their role in plant organ development and defence responses. We also surveyed small RNA sequencing studies from potato plants and presented them in a detailed report. To the best of our knowledge, phasiRNAs role in tuber development have not been reported to date. Based on the roles performed by these sRNAs in other plants, we think that phasiRNAs may fine-tune stolon-to-tuber development transition in potato. Finally, we proposed three objectives to understand the function of small RNAs in regulating stolon-to-tuber transitions. Our first objective was to perform small RNA sequencing analysis for early stolon stages in order to finalize candidate small RNAs for the study.

Chapter 2: Genome-wide identification of small RNAs from stolon-to-tuber transition stages of potato under photoperiodic conditions

Small RNA transcriptome data from the early stolon stages of potato was limited. To study the role of photoperiod during stolon-to-tuber transitions, we performed a small RNA sequencing of stolon samples from a photoperiod-sensitive potato sub-species *andigena* harvested on 4th, 7th and 10th day grown under SD and LD inductions. As one of our objectives was to identify small RNAs involved in stolon-to-tuber transitions, we conducted sRNA analysis, mainly the identification of miRNA and phasiRNAs, and studying the differential expression profiles of DE miRNAs, phasiRNAs and their predicted targets. We have performed quantitative PCRs and cleavage site mapping assay to validate differentially expressed miRNAs and the candidate target genes. Target genes were categorized based on their role in the tuberization pathway. Key findings from this study have been listed below.

1. We have identified seven conserved and twelve novel miRNAs differentially expressed (DE) at the early stolon stages under SD vs LD photoperiodic conditions. A larger number of DE miRNAs expressed under LD4 vs SD4 conditions, suggests that day length affects miRNAs' expression in the initial stolon stages (Fig. 2.7).
2. Conserved miRNAs, such as *Stu-miR479* and *Stu-miR319b-3p*, exhibited the highest expression in SD4 stolons. In contrast, *StGRAS* (a target of *miR479*) and *StGAMYB* (a target of *miR319b-3p*) showed inverse expression patterns with respect to their miRNAs. It indicated the possible role of *miR479-StGRAS* and *miR319-StGAMYB* modules during tuber development (Fig. 2.8 and 2.11) process.
3. Potato specific miRNAs, such as *n-miR147* and *n-miR 206* were induced under SD conditions (Fig. 2.9). Some of the potato specific miRNAs have also been predicted to cleave important epigenetic factors like SET domain proteins (*ASHR3* targeted by novel-miR-221). It suggests the potential role of novel miRNAs in potato development.
4. Gene Ontology (GO) analysis for the putative miRNA target genes suggested that these target genes were enriched in categories like transcription factors, phytohormone biosynthesis and signaling pathways, and light-regulated genes (Fig. 2.10).
5. We also identified 830 putative *TAS-like loci* from the potato genome. Of which, 24 were predicted to be triggered by miRNAs and could generate phased siRNAs (Table 2.5).
6. *TAS* loci, such as *TAS3* and *TAS5* were consistently found conserved in our analysis (Fig. 2.15).

7. We have validated two *TAS-like loci* located on *StTm2* and *StPHO2* transcripts, and phasiRNA produced were detected from the stolon samples (Fig. 2.14).
8. Altogether, 190 phased siRNAs were identified to be generated from 24 *TAS-like loci*. Target prediction analysis revealed that several key tuberization-related genes are the putative targets of phasiRNAs (Table 2.6).

Overall, our analysis suggested that miRNAs and phasiRNAs can target numerous genes that could be associated with tuber development pathway. A part of this work has been published in BMC Plant Biology (Kondhare, Malankar et al., 2018).

Chapter 3: PhasiRNA siRD29(-) regulates a gibberellin biosynthesis gene *StGA3ox3* during stolon-to-tuber transitions in potato

In this study, we have demonstrated a novel gene regulatory module (GRM) governing stolon-to-tuber development in potato. Reduction of gibberellin levels in stolons is a major pre-requisite for stolon-to-tuber transitions. Reduced expression of GA biosynthesis genes and increased levels of GA catabolism genes contribute to a decreased bioactive GAs in stolons. Interestingly, we observed that *StGA3ox3*, a novel GA biosynthesis gene, is induced during tuber development process, which is against the general notion. This observation led us to hypothesize that the *StGA3ox3* may be regulated by another gene regulatory mechanism. Here, we show that a GRM controlling *StGA3ox3* levels in tuber development and we propose our hypothesis for the spatio-temporal regulation of GA levels in stolon. *In silico* analysis of *StGA3ox3* promoter, followed by transient infiltration assays in the *promStGA3ox3::GUS* transgenic lines, we proved that StBEL5 (a positive regulator of tuberization and a transcription factor) induces *StGA3ox3* expression in potato. Overall, our results indicate that the Stu-miR482c-*StRGA4*-siRD29(-) module and StBEL5 transcription factor complex enable plant to fine-tune bioactive GA levels during stolon-to-tuber transitions. The salient results from this work are as follows:

1. Through transient expression assays, followed by small RNA sequencing, we establish that the Stu-miR482c-*StRGA4*-siRD29(-) gene regulatory module (GRM) is active *in planta* (Fig. 3.1 and 3.5).
2. This GRM targets a novel GA3ox isoform, *StGA3ox3* in potato, and we further show that *StGA3ox3* (a GA biosynthesis gene) could be involved in GA₃ and GA₁ biosynthesis (Fig. 3.3).

3. SiRD29(-) and *StGA3ox3* exhibit an inverse expression pattern during the stolon to swollen stolon stage and leaves under tuber-inducing short-day (SD) conditions (Fig. 3.4 and 3.6).
4. Overexpression of siRD29(-) and knockdown of *StGA3ox3* showed a similar tuber numbers. Quantitative real time PCR studies from *StRGA4-OE* and *StGA3ox3-AS* lines revealed that the *Stu-miR482c-StRGA4-siRD29(-)-StGA3ox3* GRM is functional in potato leaf and stolons (Fig. 3.15 and 3.16).
5. *In vitro* tuberization assays (with or without the GA inhibitor) and *in vivo* observation from soil-grown transgenic lines suggest that *StGA3ox3* controls the tuber-stalk development (Fig. 3.19-3.21).
6. Bioinformatic analysis of the *StGA3ox3* promoter indicates that *StGA3ox3* could be regulated by phytohormones and StBEL5 TF complex. Further, we show that *StGA3ox3* expression is induced by StBEL5 and jasmonic acid, whereas gibberellins and auxins inhibits its expression (Fig 3.22-3.25).
7. Based on the results, we demonstrate that *StGA3ox3* is regulated by two distinct mechanisms during stolon-to-tuber transition process in potato: (i) through a phasiRNA siRD29(-) -mediated post-transcriptional regulation, and (ii) the induction of *StGA3ox3* by the mobile tuberization signal, StBEL5 (Fig. 3.27).
8. We observed that this GRM is also conserved in tomato, indicating the significance of such modules in fine tuning plant developmental processes (Fig. 3.28).

A part of this work has been published in “Plant Physiology” (Malankar et al., 2023)

Chapter 4: Understanding the function of potato specific miRNA (miR72) in regulation of tuber formation in potato

We have identified several potato specific miRNAs from stolon stages with crucial tuberization genes as their putative targets. In this work, we have selected a few candidate miRNAs that were also reported in some of the previous small RNA sequencing studies (Zhang et al., 2013; Lakhotia et al., 2014; Ou et al., 2017). Based on their expression pattern under tuber inductive conditions, we have selected a highly abundant potato specific miRNA, miR72 for further study. Previous work by Ou et al. (2017) suggested that miR72 could be cold-inducible in potato. We further identified miR72 as an intronic miRNA, whose expression might be regulated by an alternate splicing (AS) mechanism. Furthermore, a sessile tuber phenotype was noticed in *miR72-OE* lines, whereas *STTM72-KD* lines had no such phenotype.

We also observed that sessile tuber phenotype of *miR72-OE* lines could be independent of photoperiod. In summary, our preliminary findings suggest the potential role of miR72 in early stolon development. This work is currently being pursued for a detailed characterization of miR72 in potato and also understanding its gene regulation mechanism. Our preliminary work in this direction has following findings.

1. Potato specific miRNA, miR72 was validated and sequence confirmed from the potato genome (Fig. 4.1-4.2).
2. Quantitative PCR analysis revealed that miR72 exhibits a abundant expression throughout potato plants, and its level decreases from stolon-to-tuber transition stages (Fig. 4.3).
3. Overexpression lines of miR72 had exhibited reduced tuber yield and root biomass. These plants also produced sessile tubers (Fig 4.6-4.7).
4. Since *miR72-OE* lines of Désirée (a day-neutral cultivar) developed sessile tubers, indicating that this phenotype could be photoperiod-independent (Fig 4.7) and miR72 might be involved in early stolon development.
5. *Cryptochrome 1b* was confirmed as a miR72 target by the cleavage site mapping assay. We could not confirm other targets, but *StIT1* expression (a key tuberization marker gene) was found to be reduced in *miR72-OE* line (Fig. 4.8), indicating that miR72 might target *StIT1* in a tissue-specific manner.
6. We also noticed that a precursor of miR72 resides within the first intron of *Profilin* gene. Expression of miR72 and its precursor decreased from stolon-to-tuber transitions, but host gene (*Profilin*) levels remain unchanged. We speculate that miR72 expression might be regulated by an alternate splicing mechanism (Fig. 4.3), only future experiments can unravel a detailed insights in this regard.

In summary, our research findings contributed in the knowledge of small RNAs regulating stolon-to-tuber transition events of potato development. This has opened up new avenues about the role of small RNAs as important regulators of tuberization. We also demonstrate the functioning of a novel phasiRNA-mediated gene regulatory module controlling stolon-to-tuber transitions in potato.

Future directions

Our investigations and interesting findings to understand the role of small RNAs in stolon-to-tuber transitions have opened-up new avenues and raised exciting questions that can be studied further. To gain further insights in this complex yet fascinating developmental transition process, the following approaches can be considered.

1. Our small RNA sequencing datasets has revealed many differentially expressed miRNAs and phasiRNAs having key tuberization genes as their putative targets, indicating their possible role in tuber development and some of the key targets along with phasiRNAs needs to be further investigated.
2. We observed that *Stu-miR482c-StRGA4-siRD29(-)-StGA3ox3* module is functional in stolon-to-tuber transitions; however, the upstream regulation of this module is yet unknown and can be explored further.
3. *MiR72-OE* lines exhibited sessile tubers with less tuber number and root biomass. Identifying miR72 targets causing this phenotype would be an interesting question to be studied in future.
4. The precursor of miR72 is located within the intronic region of *Profilin* gene and its expression pattern, indicates its possible role in tuber development. The possibilities of alternate splicing mechanism in this regard cannot be ruled out.

References

- Abelenda, J. A., Bergonzi, S., Oortwijn, M., Sonnewald, S., Du, M., Visser, R. G. F., Sonnewald, U., & Bachem, C. W. B. (2019). Source-Sink Regulation Is Mediated by Interaction of an FT Homolog with a SWEET Protein in Potato. *Current Biology*, 29(7), 1178–1186.e6. <https://doi.org/10.1016/j.cub.2019.02.018>
- Abelenda, J. A., Cruz-Oró, E., Franco-Zorrilla, J. M., & Prat, S. (2016). Potato StCONSTANS-like1 Suppresses Storage Organ Formation by Directly Activating the FT-like StSP5G Repressor. *Current Biology*, 26(7), 872–881. <https://doi.org/10.1016/j.cub.2016.01.066>
- Adenot, X., Elmayan, T., Lauressergues, D., Boutet, S., Bouché, N., Gasciolli, V., & Vaucheret, H. (2006). DRB4-Dependent TAS3 trans-Acting siRNAs Control Leaf Morphology through AGO7. *Current Biology*, 16(9), 927–932. <https://doi.org/10.1016/j.cub.2006.03.035>
- Alder, A., Jamil, M., Marzorati, M., Bruno, M., Vermathen, M., Bigler, P., Ghisla, S., Bouwmeester, H., Beyer, P., & Al-Babili, S. (2012). The Path from β -Carotene to Carlactone, a Strigolactone-Like Plant Hormone. *Science*, 335(6074), 1348–1351. <https://doi.org/10.1126/science.1218094>
- Amasino, R. M., & Michaels, S. D. (2010). The Timing of Flowering. *Plant Physiology*, 154(2), 516–520. <https://doi.org/10.1104/pp.110.161653>
- Aukerman, M. J., & Sakai, H. (2003). Regulation of Flowering Time and Floral Organ Identity by a MicroRNA and Its *APETALA2* -Like Target Genes. *The Plant Cell*, 15(11), 2730–2741. <https://doi.org/10.1105/tpc.016238>
- Axtell, M. J. (2013). Classification and Comparison of Small RNAs from Plants. *Annual Review of Plant Biology*, 64(1), 137–159. <https://doi.org/10.1146/annurev-arplant-050312-120043>
- Axtell, M. J., Jan, C., Rajagopalan, R., & Bartel, D. P. (2006). A Two-Hit Trigger for siRNA Biogenesis in Plants. *Cell*, 127(3), 565–577. <https://doi.org/10.1016/j.cell.2006.09.032>
- Baldrich, P., Bélanger, S., Kong, S., Pokhrel, S., Tamim, S., Teng, C., Schiebout, C., Gurazada, S. G. R., Gupta, P., Patel, P., Razifard, H., Nakano, M., Dusia, A., Meyers, B. C., & Frank, M. H. (2022). The evolutionary history of small RNAs in Solanaceae. *Plant Physiology*. <https://doi.org/10.1093/plphys/kiac089>
- Banerjee, A. K., Prat, S., & Hannapel, D. J. (2006). Efficient production of transgenic potato (*S. tuberosum* L. ssp. *andigena*) plants via *Agrobacterium tumefaciens*-mediated transformation. *Plant Science*, 170(4), 732–738. <https://doi.org/10.1016/j.plantsci.2005.11.007>
- Banerjee, A. K., Chatterjee, M., Yu, Y., Suh, S.-G., Miller, W. A., & Hannapel, D. J. (2007). Dynamics of a Mobile RNA of Potato Involved in a Long-Distance Signaling Pathway. *The Plant Cell*, 18(12), 3443–3457. <https://doi.org/10.1105/tpc.106.042473>
- Bhogale, S., Mahajan, A. S., Natarajan, B., Rajabhoj, M., Thulasiram, H. V., & Banerjee, A. K. (2014). *MicroRNA156*: A Potential Graft-Transmissible MicroRNA That Modulates Plant Architecture and Tuberization in *Solanum tuberosum* ssp. *andigena*. *Plant Physiology*, 164(2), 1011–1027. <https://doi.org/10.1104/pp.113.230714>
- Bonar, N., Liney, M., Zhang, R., Austin, C., Dessoly, J., Davidson, D., Stephens, J.,

- McDougall, G., Taylor, M., Bryan, G. J., & Hornyik, C. (2018). Potato miR828 Is Associated With Purple Tuber Skin and Flesh Color. *Frontiers in Plant Science*, 9. <https://doi.org/10.3389/fpls.2018.01742>
- Bou-Torrent, J., Martínez-García, J. F., García-Martínez, J. L., & Prat, S. (2011). Gibberellin A1 Metabolism Contributes to the Control of Photoperiod-Mediated Tuberization in Potato. *PLoS ONE*, 6(9), e24458. <https://doi.org/10.1371/journal.pone.0024458>
- Carrera, E., Bou, J., Garcia-Martinez, J. L., & Prat, S. (2000). Changes in GA 20-oxidase gene expression strongly affect stem length, tuber induction and tuber yield of potato plants. *The Plant Journal*, 22(3), 247–256. <https://doi.org/10.1046/j.1365-313x.2000.00736.x>
- Chailakhyan MKh, Yanina LI, Davedzhiyan AG, & Lotova GN. (1981). Photoperiodism and tuberization with grafting of tobacco on potato. *Doklady Akademii Nauk SSSR*, 257.
- Chen, H., Banerjee, A. K., & Hannapel, D. J. (2004). The tandem complex of BEL and KNOX partners is required for transcriptional repression of *ga20ox1*. *The Plant Journal*, 38(2), 276–284. <https://doi.org/10.1111/j.1365-313X.2004.02048.x>
- Chen, H., Rosin, F. M., Prat, S., & Hannapel, D. J. (2003). Interacting Transcription Factors from the Three-Amino Acid Loop Extension Superclass Regulate Tuber Formation. *Plant Physiology*, 132(3), 1391–1404. <https://doi.org/10.1104/pp.103.022434>
- Chincinska, I. A., Liesche, J., Krügel, U., Michalska, J., Geigenberger, P., Grimm, B., & Kühn, C. (2008). Sucrose Transporter StSUT4 from Potato Affects Flowering, Tuberization, and Shade Avoidance Response. *Plant Physiology*, 146(2), 323–324. <https://doi.org/10.1104/pp.107.112334>
- Chitwood, D. H., Nogueira, F. T. S., Howell, M. D., Montgomery, T. A., Carrington, J. C., & Timmermans, M. C. P. (2009). Pattern formation via small RNA mobility. *Genes & Development*, 23(5), 549–554. <https://doi.org/10.1101/gad.177009>
- Cho, S. K., Sharma, P., Butler, N. M., Kang, I.-H., Shah, S., Rao, A. G., & Hannapel, D. J. (2015). Polypyrimidine tract-binding proteins of potato mediate tuberization through an interaction with *StBEL5* RNA. *Journal of Experimental Botany*, 66(21), 6835–6847. <https://doi.org/10.1093/jxb/erv389>
- Chow, C.-N., Lee, T.-Y., Hung, Y.-C., Li, G.-Z., Tseng, K.-C., Liu, Y.-H., Kuo, P.-L., Zheng, H.-Q., & Chang, W.-C. (2019). PlantPAN3.0: a new and updated resource for reconstructing transcriptional regulatory networks from ChIP-seq experiments in plants. *Nucleic Acids Research*, 47(D1), D1155–D1163. <https://doi.org/10.1093/nar/gky1081>
- Dai, X., Zhuang, Z., & Zhao, P. X. (2018). psRNATarget: a plant small RNA target analysis server (2017 release). *Nucleic Acids Research*, 46(W1), W49–W54. <https://doi.org/10.1093/nar/gky316>
- Dong, Q., Hu, B., & Zhang, C. (2022). microRNAs and Their Roles in Plant Development. *Frontiers in Plant Science*, 13. <https://doi.org/10.3389/fpls.2022.824240>
- Eviatar-Ribak, T., Shalit-Kaneh, A., Chappell-Maor, L., Amsellem, Z., Eshed, Y., & Lifschitz, E. (2013). A Cytokinin-Activating Enzyme Promotes Tuber Formation in Tomato. *Current Biology*, 23(12), 1057–1064. <https://doi.org/10.1016/j.cub.2013.04.061>
- Ewing EE, & Struik PC. (1992). *Tuber formation in potato: induction, initiation, and growth*. (Vol. 14, pp. 89–108).

- Fahlgren, N., Montgomery, T. A., Howell, M. D., Allen, E., Dvorak, S. K., Alexander, A. L., & Carrington, J. C. (2006). Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA Affects Developmental Timing and Patterning in Arabidopsis. *Current Biology*, 16(9), 939–944. <https://doi.org/10.1016/j.cub.2006.03.065>
- Fei, Q., Xia, R., & Meyers, B. C. (2013). Phased, Secondary, Small Interfering RNAs in Posttranscriptional Regulatory Networks. *The Plant Cell*, 25(7), 2400–2415. <https://doi.org/10.1105/tpc.113.114652>
- Fukudome, A., & Fukuhara, T. (2017). Plant dicer-like proteins: double-stranded RNA-cleaving enzymes for small RNA biogenesis. *Journal of Plant Research*, 130(1), 33–44. <https://doi.org/10.1007/s10265-016-0877-1>
- Genome sequence and analysis of the tuber crop potato. (2011). *Nature*, 475(7355), 189–195. <https://doi.org/10.1038/nature10158>
- Gibson, S. I. (2005). Control of plant development and gene expression by sugar signaling. *Current Opinion in Plant Biology*, 8(1), 93–102. <https://doi.org/10.1016/j.pbi.2004.11.003>
- Gregory, L. E. (1956). Some Factors for Tuberization in the Potato Plant. *American Journal of Botany*, 43(4), 281. <https://doi.org/10.2307/2438945>
- Guan, X., Pang, M., Nah, G., Shi, X., Ye, W., Stelly, D. M., & Chen, Z. J. (2014). miR828 and miR858 regulate homoeologous MYB2 gene functions in Arabidopsis trichome and cotton fibre development. *Nature Communications*, 5(1), 3050. <https://doi.org/10.1038/ncomms4050>
- Guo, G., Liu, X., Sun, F., Cao, J., Huo, N., Wuda, B., Xin, M., Hu, Z., Du, J., Xia, R., Rossi, V., Peng, H., Ni, Z., Sun, Q., & Yao, Y. (2018). Wheat miR9678 Affects Seed Germination by Generating Phased siRNAs and Modulating Absciscic Acid/Gibberellin Signaling. *The Plant Cell*, 30(4), 796–814. <https://doi.org/10.1105/tpc.17.00842>
- Ha, M., & Kim, V. N. (2014). Regulation of microRNA biogenesis. *Nature Reviews Molecular Cell Biology*, 15(8), 509–524. <https://doi.org/10.1038/nrm3838>
- Hannapel, D. J., Sharma, P., Lin, T., & Banerjee, A. K. (2017). The Multiple Signals That Control Tuber Formation. *Plant Physiology*, 174(2), 845–856. <https://doi.org/10.1104/pp.17.00272>
- Higo, K., Ugawa, Y., Iwamoto, M., & Korenaga, T. (1999). Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Research*, 27(1), 297–300. <https://doi.org/10.1093/nar/27.1.297>
- Jackson, S. D. (2009). Plant responses to photoperiod. *New Phytologist*, 181(3), 517–531. <https://doi.org/10.1111/j.1469-8137.2008.02681.x>
- Jackson, S. D., James, P., Prat, S., & Thomas, B. (1998). Phytochrome B Affects the Levels of a Graft-Transmissible Signal Involved in Tuberization1. *Plant Physiology*, 117(1), 29–32. <https://doi.org/10.1104/pp.117.1.29>
- Jing, S., Sun, X., Yu, L., Wang, E., Cheng, Z., Liu, H., Jiang, P., Qin, J., Begum, S., & Song, B. (2022). Transcription factor StABI5-like 1 binding to the FLOWERING LOCUS T homologs promotes early maturity in potato. *Plant Physiology*, 189(3), 1677–1693. <https://doi.org/10.1093/plphys/kiac098>
- Kidner, C. A., & Martienssen, R. A. (2004). Spatially restricted microRNA directs leaf polarity

- through ARGONAUTE1. *Nature*, 428(6978), 81–84. <https://doi.org/10.1038/nature02366>
- Kloosterman, B., Abelenda, J. A., Gomez, M. del M. C., Oortwijn, M., de Boer, J. M., Kowitwanich, K., Horvath, B. M., van Eck, H. J., Smaczniak, C., Prat, S., Visser, R. G. F., & Bachem, C. W. B. (2013). Naturally occurring allele diversity allows potato cultivation in northern latitudes. *Nature*, 495(7440), 246–250. <https://doi.org/10.1038/nature11912>
- Kloosterman, B., De Koeijer, D., Griffiths, R., Flinn, B., Steuernagel, B., Scholz, U., Sonnewald, S., Sonnewald, U., Bryan, G. J., Prat, S., Bánfalvi, Z., Hammond, J. P., Geigenberger, P., Nielsen, K. L., Visser, R. G. F., & Bachem, C. W. B. (2008). Genes driving potato tuber initiation and growth: identification based on transcriptional changes using the POCI array. *Functional & Integrative Genomics*, 8(4), 329–340. <https://doi.org/10.1007/s10142-008-0083-x>
- Kloosterman, B., Vorst, O., Hall, R. D., Visser, R. G. F., & Bachem, C. W. (2005). Tuber on a chip: differential gene expression during potato tuber development. *Plant Biotechnology Journal*, 3(5), 505–519. <https://doi.org/10.1111/j.1467-7652.2005.00141.x>
- Knauer, S., Holt, A. L., Rubio-Somoza, I., Tucker, E. J., Hinze, A., Pisch, M., Javelle, M., Timmermans, M. C., Tucker, M. R., & Laux, T. (2013). A Protodermal miR394 Signal Defines a Region of Stem Cell Competence in the Arabidopsis Shoot Meristem. *Developmental Cell*, 24(2), 125–132. <https://doi.org/10.1016/j.devcel.2012.12.009>
- Kondhare, K. R., Kumar, A., Patil, N. S., Malankar, N. N., Saha, K., & Banerjee, A. K. (2021). Development of aerial and belowground tubers in potato is governed by photoperiod and epigenetic mechanism. *Plant Physiology*, 187(3), 1071–1086. <https://doi.org/10.1093/plphys/kiab409>
- Križnik, M., Petek, M., Dobnik, D., Ramšak, Ž., Baebler, Š., Pollmann, S., Kreuze, J. F., Žel, J., & Gruden, K. (2017). Salicylic Acid Perturbs sRNA-Gibberellin Regulatory Network in Immune Response of Potato to Potato virus Y Infection. *Frontiers in Plant Science*, 8. <https://doi.org/10.3389/fpls.2017.02192>
- Kumar, A., Kondhare, K. R., Vetel, P. V., & Banerjee, A. K. (2020). PcG Proteins MSI1 and BMI1 Function Upstream of miR156 to Regulate Aerial Tuber Formation in Potato. *Plant Physiology*, 182(1), 185–203. <https://doi.org/10.1104/pp.19.00416>
- KUMAR, D., & WAREING, P. F. (1974). STUDIES ON TUBERIZATION OF SOLANUM ANDIGENA. II. GROWTH HORMONES AND TUBERIZATION. *New Phytologist*, 73(5), 833–840. <https://doi.org/10.1111/j.1469-8137.1974.tb01311.x>
- Lehretz, G. G., Sonnewald, S., Hornyik, C., Corral, J. M., & Sonnewald, U. (2019). Post-transcriptional Regulation of FLOWERING LOCUS T Modulates Heat-Dependent Source-Sink Development in Potato. *Current Biology*, 29(10), 1614–1624.e3. <https://doi.org/10.1016/j.cub.2019.04.027>
- Lian, H., Wang, L., Ma, N., Zhou, C.-M., Han, L., Zhang, T.-Q., & Wang, J.-W. (2021). Redundant and specific roles of individual MIR172 genes in plant development. *PLOS Biology*, 19(2), e3001044. <https://doi.org/10.1371/journal.pbio.3001044>
- Lin, T., Sharma, P., Gonzalez, D. H., Viola, I. L., & Hannapel, D. J. (2013). The Impact of the Long-Distance Transport of a *BEL1* -Like Messenger RNA on Development. *Plant Physiology*, 161(2), 760–772. <https://doi.org/10.1104/pp.112.209429>
- Lin, Y., Lin, L., Lai, R., Liu, W., Chen, Y., Zhang, Z., XuHan, X., & Lai, Z. (2015).

- MicroRNA390-Directed TAS3 Cleavage Leads to the Production of tasiRNA-ARF3/4 During Somatic Embryogenesis in *Dimocarpus longan* Lour. *Frontiers in Plant Science*, 6. <https://doi.org/10.3389/fpls.2015.01119>
- Liu, J., Osbourn, A., & Ma, P. (2015). MYB Transcription Factors as Regulators of Phenylpropanoid Metabolism in Plants. *Molecular Plant*, 8(5), 689–708. <https://doi.org/10.1016/j.molp.2015.03.012>
- Liu, P.-P., Montgomery, T. A., Fahlgren, N., Kasschau, K. D., Nonogaki, H., & Carrington, J. C. (2007). Repression of AUXIN RESPONSE FACTOR10 by microRNA160 is critical for seed germination and post-germination stages. *The Plant Journal*, 52(1), 133–146. <https://doi.org/10.1111/j.1365-313X.2007.03218.x>
- Liu, Y., Teng, C., Xia, R., & Meyers, B. C. (2020). PhasiRNAs in Plants: Their Biogenesis, Genic Sources, and Roles in Stress Responses, Development, and Reproduction. *The Plant Cell*, 32(10), 3059–3080. <https://doi.org/10.1105/TPC.20.00335>
- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods*, 25(4), 402–408. <https://doi.org/10.1006/meth.2001.1262>
- López-Ráez, J. A., Charnikhova, T., Gómez-Roldán, V., Matusova, R., Kohlen, W., De Vos, R., Verstappen, F., Puech-Pages, V., Bécard, G., Mulder, P., & Bouwmeester, H. (2008). Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. *New Phytologist*, 178(4), 863–874. <https://doi.org/10.1111/j.1469-8137.2008.02406.x>
- Luisi, A., Lorenzi, R., & Sorce, C. (2011). Strigolactone may interact with gibberellin to control apical dominance in pea (*Pisum sativum*). *Plant Growth Regulation*, 65(2), 415–419. <https://doi.org/10.1007/s10725-011-9603-0>
- Mahajan, A., Bhogale, S., Kang, I. H., Hannapel, D. J., & Banerjee, A. K. (2012). The mRNA of a Knotted1-like transcription factor of potato is phloem mobile. *Plant Molecular Biology*, 79(6), 595–608. <https://doi.org/10.1007/s11103-012-9931-0>
- Marin, E., Jouannet, V., Herz, A., Lokerse, A. S., Weijers, D., Vaucheret, H., Nussaume, L., Crespi, M. D., & Maizel, A. (2010). miR390, *Arabidopsis* TAS3 tasiRNAs, and Their AUXIN RESPONSE FACTOR Targets Define an Autoregulatory Network Quantitatively Regulating Lateral Root Growth. *The Plant Cell*, 22(4), 1104–1117. <https://doi.org/10.1105/tpc.109.072553>
- Martin, A., Adam, H., Díaz-Mendoza, M., Żurczak, M., González-Schain, N. D., & Suárez-López, P. (2009). Graft-transmissible induction of potato tuberization by the microRNA *miR172*. *Development*, 136(17), 2873–2881. <https://doi.org/10.1242/dev.031658>
- Martínez-García, J. F., Virgós-Soler, A., & Prat, S. (2002). Control of photoperiod-regulated tuberization in potato by the *Arabidopsis* flowering-time gene *CONSTANS*. *Proceedings of the National Academy of Sciences*, 99(23), 15211–15216. <https://doi.org/10.1073/pnas.222390599>
- Mauk, C. S., & Langille, A. R. (1978). Physiology of Tuberization in *Solanum tuberosum* L. *Plant Physiology*, 62(3), 438–442. <https://doi.org/10.1104/pp.62.3.438>
- Minakuchi, K., Kameoka, H., Yasuno, N., Umehara, M., Luo, L., Kobayashi, K., Hanada, A., Ueno, K., Asami, T., Yamaguchi, S., & Kyoizuka, J. (2010). FINE CULM1 (FC1) Works

- Downstream of Strigolactones to Inhibit the Outgrowth of Axillary Buds in Rice. *Plant and Cell Physiology*, 51(7), 1127–1135. <https://doi.org/10.1093/pcp/pcq083>
- Luisi, A., Lorenzi, R., & Sorce, C. (2011). Strigolactone may interact with gibberellin to control apical dominance in pea (*Pisum sativum*). *Plant Growth Regulation*, 65(2), 415–419. <https://doi.org/10.1007/s10725-011-9603-0>
- Navarro, C., Abellenda, J. A., Cruz-Oró, E., Cuéllar, C. A., Tamaki, S., Silva, J., Shimamoto, K., & Prat, S. (2011). Control of flowering and storage organ formation in potato by FLOWERING LOCUS T. *Nature*, 478(7367), 119–122. <https://doi.org/10.1038/nature10431>
- Nicolas, M., Torres-Pérez, R., Wahl, V., Cruz-Oró, E., Rodríguez-Buey, M. L., Zamarreño, A. M., Martín-Jouve, B., García-Mina, J. M., Oliveros, J. C., Prat, S., & Cubas, P. (2022). Spatial control of potato tuberization by the TCP transcription factor BRANCHED1b. *Nature Plants*, 8(3), 281–294. <https://doi.org/10.1038/s41477-022-01112-2>
- Ou, Y., Liu, X., Xie, C., Zhang, H., Lin, Y., Li, M., Song, B., & Liu, J. (2015). Genome-Wide Identification of microRNAs and Their Targets in Cold-Stored Potato Tubers by Deep Sequencing and Degradome Analysis. *Plant Molecular Biology Reporter*, 33(3), 584–597. <https://doi.org/10.1007/s11105-014-0771-8>
- PALMER, C. E., & SMITH, O. E. (1970). Effect of kinetin on tuber formation on isolated stolons of *Solanum tuberosum* L. cultured in vitro. *Plant and Cell Physiology*, 11(2), 303–314. <https://doi.org/10.1093/oxfordjournals.pcp.a074511>
- Pasare, S. A., Ducreux, L. J. M., Morris, W. L., Campbell, R., Sharma, S. K., Roumeliotis, E., Kohlen, W., Krol, S., Bramley, P. M., Roberts, A. G., Fraser, P. D., & Taylor, M. A. (2013). The role of the potato (*Solanum tuberosum*) CCD8 gene in stolon and tuber development. *New Phytologist*, 198(4), 1108–1120. <https://doi.org/10.1111/nph.12217>
- Pelacho, A. M., & Mingo-Castel, A. M. (1991). Effects of photoperiod on kinetin-induced tuberization of isolated potato stolons cultured in vitro. *American Potato Journal*, 68(8), 533–541. <https://doi.org/10.1007/BF02853770>
- Piazza, P., Jasinski, S., & Tsiantis, M. (2005). Evolution of leaf developmental mechanisms. *New Phytologist*, 167(3), 693–710. <https://doi.org/10.1111/j.1469-8137.2005.01466.x>
- Picó, S., Ortiz-Marchena, M. I., Merini, W., & Calonje, M. (2015). Deciphering the Role of POLYCOMB REPRESSIVE COMPLEX1 Variants in Regulating the Acquisition of Flowering Competence in Arabidopsis. *Plant Physiology*, 168(4), 1286–1297. <https://doi.org/10.1104/pp.15.00073>
- Preston, J. C., & Hileman, L. C. (2013). Functional Evolution in the Plant SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) Gene Family. *Frontiers in Plant Science*, 4. <https://doi.org/10.3389/fpls.2013.00080>
- Quarrie, S. A. (1982). Droopy: a wilted mutant of potato deficient in abscisic acid. *Plant, Cell and Environment*, 5(1), 23–26. <https://doi.org/10.1111/1365-3040.ep11587567>
- Reinhart, B. J., Weinstein, E. G., Rhoades, M. W., Bartel, B., & Bartel, D. P. (2002). MicroRNAs in plants. *Genes & Development*, 16(13), 1616–1626. <https://doi.org/10.1101/gad.1004402>
- Rodríguez-Falcón, M., Bou, J., & Prat, S. (2006). SEASONAL CONTROL OF TUBERIZATION IN POTATO: Conserved Elements with the Flowering Response.

- Annual Review of Plant Biology*, 57(1), 151–180.
<https://doi.org/10.1146/annurev.arplant.57.032905.105224>
- Rogers, K., & Chen, X. (2013). Biogenesis, Turnover, and Mode of Action of Plant MicroRNAs. *The Plant Cell*, 25(7), 2383–2399. <https://doi.org/10.1105/tpc.113.113159>
- Rosin, F. M., Hart, J. K., Horner, H. T., Davies, P. J., & Hannapel, D. J. (2003). Overexpression of a *Knotted* -Like Homeobox Gene of Potato Alters Vegetative Development by Decreasing Gibberellin Accumulation. *Plant Physiology*, 132(1), 106–117. <https://doi.org/10.1104/pp.102.015560>
- Roumeliotis, E., Kloosterman, B., Oortwijn, M., Kohlen, W., Bouwmeester, H. J., Bachem, C. W. B., & Visser, R. G. F. (2023). Over-expression of a YUCCA-Like Gene Results in Altered Shoot and Stolon Branching and Reduced Potato Tuber Size. *Potato Research*, 66(1), 67–84. <https://doi.org/10.1007/s11540-022-09572-x>
- Roumeliotis, E., Kloosterman, B., Oortwijn, M., Kohlen, W., Bouwmeester, H. J., Visser, R. G. F., & Bachem, C. W. B. (2012). The effects of auxin and strigolactones on tuber initiation and stolon architecture in potato. *Journal of Experimental Botany*, 63(12), 4539–4547. <https://doi.org/10.1093/jxb/ers132>
- Roumeliotis, E., Kloosterman, B., Oortwijn, M., Lange, T., Visser, R. G. F., & Bachem, C. W. B. (2013). Down regulation of StGA3ox genes in potato results in altered GA content and affect plant and tuber growth characteristics. *Journal of Plant Physiology*, 170(14), 1228–1234. <https://doi.org/10.1016/j.jplph.2013.04.003>
- Roumeliotis, E., Kloosterman, B., Oortwijn, M., Visser, R. G. F., & Bachem, C. W. B. (2013). The PIN family of proteins in potato and their putative role in tuberization. *Frontiers in Plant Science*, 4. <https://doi.org/10.3389/fpls.2013.00524>
- Roumeliotis, E., Visser, R. G. F., & Bachem, C. W. B. (2012). A crosstalk of auxin and GA during tuber development. *Plant Signaling & Behavior*, 7(10), 1360–1363. <https://doi.org/10.4161/psb.21515>
- Santin, F., Bhogale, S., Fantino, E., Grandellis, C., Banerjee, A. K., & Ulloa, R. M. (2017). *Solanum tuberosum* StCDPK1 is regulated by miR390 at the posttranscriptional level and phosphorylates the auxin efflux carrier StPIN4 in vitro, a potential downstream target in potato development. *Physiologia Plantarum*, 159(2), 244–261. <https://doi.org/10.1111/ppl.12517>
- Sarkar, D. (2010). Photoperiodic inhibition of potato tuberization: an update. *Plant Growth Regulation*, 62(2), 117–125. <https://doi.org/10.1007/s10725-010-9502-9>
- Schoof, H., Lenhard, M., Haecker, A., Mayer, K. F. ., Jürgens, G., & Laux, T. (2000). The Stem Cell Population of Arabidopsis Shoot Meristems Is Maintained by a Regulatory Loop between the CLAVATA and WUSCHEL Genes. *Cell*, 100(6), 635–644. [https://doi.org/10.1016/S0092-8674\(00\)80700-X](https://doi.org/10.1016/S0092-8674(00)80700-X)
- Schwach, F., Vaistij, F. E., Jones, L., & Baulcombe, D. C. (2005). An RNA-Dependent RNA Polymerase Prevents Meristem Invasion by Potato Virus X and Is Required for the Activity But Not the Production of a Systemic Silencing Signal. *Plant Physiology*, 138(4), 1842–1852. <https://doi.org/10.1104/pp.105.063537>
- Sharma, P., Lin, T., & Hannapel, D. J. (2016). Targets of the StBEL5 Transcription Factor Include the FT Ortholog StSP6A. *Plant Physiology*, 170(1), 310–324.

<https://doi.org/10.1104/pp.15.01314>

- Shivaprasad, P. V., Chen, H.-M., Patel, K., Bond, D. M., Santos, B. A. C. M., & Baulcombe, D. C. (2012). A MicroRNA Superfamily Regulates Nucleotide Binding Site–Leucine-Rich Repeats and Other mRNAs. *The Plant Cell*, 24(3), 859–874. <https://doi.org/10.1105/tpc.111.095380>
- Singh, A., Gautam, V., Singh, S., Sarkar Das, S., Verma, S., Mishra, V., Mukherjee, S., & Sarkar, A. K. (2018). Plant small RNAs: advancement in the understanding of biogenesis and role in plant development. *Planta*, 248(3), 545–558. <https://doi.org/10.1007/s00425-018-2927-5>
- Takahashi, K., Fujino, K., Kikuta, Y., & Koda, Y. (1994). Expansion of potato cells in response to jasmonic acid. *Plant Science*, 100(1), 3–8. [https://doi.org/10.1016/0168-9452\(94\)90127-9](https://doi.org/10.1016/0168-9452(94)90127-9)
- Tang, G., Yan, J., Gu, Y., Qiao, M., Fan, R., Mao, Y., & Tang, X. (2012). Construction of short tandem target mimic (STTM) to block the functions of plant and animal microRNAs. *Methods*, 58(2), 118–125. <https://doi.org/10.1016/j.ymeth.2012.10.006>
- Tang, D., Jia, Y., Zhang, J., Li, H., Cheng, L., Wang, P., Bao, Z., Liu, Z., Feng, S., Zhu, X., Li, D., Zhu, G., Wang, H., Zhou, Y., Zhou, Y., Bryan, G. J., Buell, C. R., Zhang, C., & Huang, S. (2022). Genome evolution and diversity of wild and cultivated potatoes. *Nature*, 606(7914), 535–541. <https://doi.org/10.1038/s41586-022-04822-x>
- Taoka, K., Ohki, I., Tsuji, H., Furuita, K., Hayashi, K., Yanase, T., Yamaguchi, M., Nakashima, C., Purwestri, Y. A., Tamaki, S., Ogaki, Y., Shimada, C., Nakagawa, A., Kojima, C., & Shimamoto, K. (2011). 14-3-3 proteins act as intracellular receptors for rice Hd3a florigen. *Nature*, 476(7360), 332–335. <https://doi.org/10.1038/nature10272>
- Teale, W. D., Paponov, I. A., & Palme, K. (2006). Auxin in action: signalling, transport and the control of plant growth and development. *Nature Reviews Molecular Cell Biology*, 7(11), 847–859. <https://doi.org/10.1038/nrm2020>
- Teo, C.-J., Takahashi, K., Shimizu, K., Shimamoto, K., & Taoka, K. (2016). Potato Tuber Induction is Regulated by Interactions Between Components of a Tuberigen Complex. *Plant and Cell Physiology*, pcw197. <https://doi.org/10.1093/pcp/pcw197>
- Tirumalai, V., Swetha, C., Nair, A., Pandit, A., & Shivaprasad, P. V. (2019). miR828 and miR858 regulate VvMYB114 to promote anthocyanin and flavonol accumulation in grapes. *Journal of Experimental Botany*, 70(18), 4775–4792. <https://doi.org/10.1093/jxb/erz264>
- Varkonyi-Gasic, E., Wu, R., Wood, M., Walton, E. F., & Hellens, R. P. (2007). Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant Methods*, 3(1), 12. <https://doi.org/10.1186/1746-4811-3-12>
- Voinnet, O. (2009). Origin, Biogenesis, and Activity of Plant MicroRNAs. *Cell*, 136(4), 669–687. <https://doi.org/10.1016/j.cell.2009.01.046>
- Werner, H. O. (1934). The effect of a controlled nitrogen supply with different temperatures and photoperiods upon the development of the potato plant. *The American Potato Journal*, 274–280.
- Wu, G., & Poethig, R. S. (2006). Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3. *Development*, 133(18), 3539–3547.

<https://doi.org/10.1242/dev.02521>

- Xia, R., Zhu, H., An, Y., Beers, E. P., & Liu, Z. (2012). Apple miRNAs and tasiRNAs with novel regulatory networks. *Genome Biology*, 13(6), R47. <https://doi.org/10.1186/gb-2012-13-6-r47>
- Xu, M., Hu, T., Smith, M. R., & Poethig, R. S. (2016). Epigenetic Regulation of Vegetative Phase Change in Arabidopsis. *The Plant Cell*, 28(1), 28–41. <https://doi.org/10.1105/tpc.15.00854>
- Xu, X., van Lammeren, A. A. M., Vermeer, E., & Vreugdenhil, D. (1998). The Role of Gibberellin, Absciscic Acid, and Sucrose in the Regulation of Potato Tuber Formation in Vitro1. *Plant Physiology*, 117(2), 575–584. <https://doi.org/10.1104/pp.117.2.575>
- Xu, X., Vreugdenhil, D., & Lammeren, A. A. M. v. (1998). Cell division and cell enlargement during potato tuber formation. *Journal of Experimental Botany*, 49(320), 573–582. <https://doi.org/10.1093/jxb/49.320.573>
- Yan, K., Liu, P., Wu, C.-A., Yang, G.-D., Xu, R., Guo, Q.-H., Huang, J.-G., & Zheng, C.-C. (2012). Stress-Induced Alternative Splicing Provides a Mechanism for the Regulation of MicroRNA Processing in Arabidopsis thaliana. *Molecular Cell*, 48(4), 521–531. <https://doi.org/10.1016/j.molcel.2012.08.032>
- Yang, T., Wang, Y., Teotia, S., Wang, Z., Shi, C., Sun, H., Gu, Y., Zhang, Z., & Tang, G. (2019). The interaction between miR160 and miR165/166 in the control of leaf development and drought tolerance in Arabidopsis. *Scientific Reports*, 9(1), 2832. <https://doi.org/10.1038/s41598-019-39397-7>
- Yifhar, T., Pekker, I., Peled, D., Friedlander, G., Pistunov, A., Sabban, M., Wachsman, G., Alvarez, J. P., Amsellem, Z., & Eshed, Y. (2012). Failure of the Tomato *Trans*-Acting Short Interfering RNA Program to Regulate AUXIN RESPONSE FACTOR3 and ARF4 Underlies the Wiry Leaf Syndrome . *The Plant Cell*, 24(9), 3575–3589. <https://doi.org/10.1105/tpc.112.100222>
- Zeevaart, J. A. D. (2006). Florigen Coming of Age after 70 Years. *The Plant Cell*, 18(8), 1783–1789. <https://doi.org/10.1105/tpc.106.043513>
- Zhang, H., Xia, R., Meyers, B. C., & Walbot, V. (2015). Evolution, functions, and mysteries of plant ARGONAUTE proteins. *Current Opinion in Plant Biology*, 27, 84–90. <https://doi.org/10.1016/j.pbi.2015.06.011>
- Zhang, L., Xiang, Y., Chen, S., Shi, M., Jiang, X., He, Z., & Gao, S. (2022). Mechanisms of MicroRNA Biogenesis and Stability Control in Plants. *Frontiers in Plant Science*, 13. <https://doi.org/10.3389/fpls.2022.844149>
- Zhang, R., Marshall, D., Bryan, G. J., & Hornyik, C. (2013). Identification and Characterization of miRNA Transcriptome in Potato by High-Throughput Sequencing. *PLoS ONE*, 8(2), e57233. <https://doi.org/10.1371/journal.pone.0057233>
- Zhang, X., Campbell, R., Ducreux, L. J. M., Morris, J., Hedley, P. E., Mellado-Ortega, E., Roberts, A. G., Stephens, J., Bryan, G. J., Torrance, L., Chapman, S. N., Prat, S., & Taylor, M. A. (2020). TERMINAL FLOWER-1/CENTRORADIALIS inhibits tuberisation via protein interaction with the tuberigen activation complex. *The Plant Journal*, 103(6), 2263–2278. <https://doi.org/10.1111/tpj.14898>
- Zhang, Y., Waseem, M., Zeng, Z., Xu, J., Chen, C., Liu, Y., Zhai, J., & Xia, R. (2022).

- MicroRNA482/2118, a miRNA superfamily essential for both disease resistance and plant development. *New Phytologist*, 233(5), 2047–2057. <https://doi.org/10.1111/nph.17853>
- Zhou, T., Song, B., Liu, T., Shen, Y., Dong, L., Jing, S., Xie, C., & Liu, J. (2019). Phytochrome F plays critical roles in potato photoperiodic tuberization. *The Plant Journal*, 98(1), 42–54. <https://doi.org/10.1111/tpj.14198>
- Zhu, H., Hu, F., Wang, R., Zhou, X., Sze, S.-H., Liou, L. W., Barefoot, A., Dickman, M., & Zhang, X. (2011). Arabidopsis Argonaute10 Specifically Sequesters miR166/165 to Regulate Shoot Apical Meristem Development. *Cell*, 145(2), 242–256. <https://doi.org/10.1016/j.cell.2011.03.024>
- Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research*, 31(13), 3406–3415. <https://doi.org/10.1093/nar/gkg595>

Annexure I

OXFORD UNIVERSITY PRESS LICENSE TERMS AND CONDITIONS

Jul 25, 2023

This Agreement between Ms. Nilam Malankar ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number	5595911150347
License date	Jul 25, 2023
Licensed content publisher	Oxford University Press
Licensed content publication	Plant Physiology
Licensed content title	Development of aerial and belowground tubers in potato is governed by photoperiod and epigenetic mechanism
Licensed content author	Kondhare, Kirtikumar R; Kumar, Amit
Licensed content date	Sep 7, 2021
Type of Use	Thesis/Dissertation
Institution name	
Title of your work	Investigating the functions of small non-coding RNAs in stolon-to-tuber transition stages of potato development
Publisher of your work	IISER Pune
Expected publication date	Aug 2023
Permissions cost	0.00 USD

Value added tax 0.00 USD

Total 0.00 USD

Title Investigating the functions of small non-coding RNAs in stolon-to-tuber transition stages of potato development

Institution name IISER Pune

Expected presentation date Aug 2023

Portions Figure 1, Table 1

Ms. Nilam Malankar
IISER Pune Hostel 3

Requestor Location Pashan Pune
Pune, Maharashtra 411008
India
Attn: Ms. Nilam Malankar

Publisher Tax ID GB125506730

Total 0.00 USD

Terms and Conditions

**STANDARD TERMS AND CONDITIONS FOR REPRODUCTION OF MATERIAL
FROM AN OXFORD UNIVERSITY PRESS JOURNAL**

1. Use of the material is restricted to the type of use specified in your order details.
2. This permission covers the use of the material in the English language in the following territory: world. If you have requested additional permission to translate this material, the terms and conditions of this reuse will be set out in clause 12.
3. This permission is limited to the particular use authorized in (1) above and does not allow you to sanction its use elsewhere in any other format other than specified above, nor does it apply to quotations, images, artistic works etc that have been reproduced from other sources which may be part of the material to be used.
4. No alteration, omission or addition is made to the material without our written consent. Permission must be re-cleared with Oxford University Press if/when you decide to reprint.

ELSEVIER LICENSE TERMS AND CONDITIONS

Jul 25, 2023

This Agreement between Ms. Nilam Malankar ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	5596090891538
License date	Jul 25, 2023
Licensed Content Publisher	Elsevier
Licensed Content Publication	Cell
Licensed Content Title	Origin, Biogenesis, and Activity of Plant MicroRNAs
Licensed Content Author	Olivier Voinnet
Licensed Content Date	Feb 20, 2009
Licensed Content Volume	136
Licensed Content Issue	4
Licensed Content Pages	19
Start Page	669
End Page	687
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations

Number of figures/tables/illustrations 1

Format both print and electronic

Are you the author of this Elsevier article? No

Will you be translating? No

Title Investigating the functions of small non-coding RNAs in stolon-to-tuber transition stages of potato development

Institution name IISER Pune

Expected presentation date Aug 2023

Portions Figure 1

Requestor Location Ms. Nilam Malankar
IISER Pune Hostel 3
Pashan Pune
Pune, Maharashtra 411008
India
Attn: Ms. Nilam Malankar

Publisher Tax ID GB 494 6272 12

Total 0.00 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your RightsLink account and that are available at any time at <https://myaccount.copyright.com>).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

OXFORD UNIVERSITY PRESS LICENSE TERMS AND CONDITIONS

Jul 25, 2023

This Agreement between Ms. Nilam Malankar ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number 5596100465248

License date Jul 25, 2023

Licensed content publisher Oxford University Press

Licensed content publication The Plant Cell

Licensed content title PhasiRNAs in Plants: Their Biogenesis, Genic Sources, and Roles in Stress Responses, Development, and Reproduction

Licensed content author Liu, Yuanlong; Teng, Chong

Licensed content date Aug 18, 2020

Type of Use Thesis/Dissertation

Institution name

Title of your work Investigating the functions of small non-coding RNAs in stolon-to-tuber transition stages of potato development

Publisher of your work IISER Pune

Expected publication date Aug 2023

Permissions cost 0.00 USD

Value added tax 0.00 USD

Total 0.00 USD

Title Investigating the functions of small non-coding RNAs in stolon-to-tuber transition stages of potato development

Institution name IISER Pune

Expected presentation date Aug 2023

Portions Figure1, 3

Ms. Nilam Malankar
IISER Pune Hostel 3

Requestor Location Pashan Pune
Pune, Maharashtra 411008
India
Attn: Ms. Nilam Malankar

Publisher Tax ID GB125506730

Total 0.00 USD

Terms and Conditions

**STANDARD TERMS AND CONDITIONS FOR REPRODUCTION OF MATERIAL
FROM AN OXFORD UNIVERSITY PRESS JOURNAL**

1. Use of the material is restricted to the type of use specified in your order details.
2. This permission covers the use of the material in the English language in the following territory: world. If you have requested additional permission to translate this material, the terms and conditions of this reuse will be set out in clause 12.
3. This permission is limited to the particular use authorized in (1) above and does not allow you to sanction its use elsewhere in any other format other than specified above, nor does it apply to quotations, images, artistic works etc that have been reproduced from other sources which may be part of the material to be used.



Marketplace

This is a License Agreement between Nilam Malankar ("User") and Copyright Clearance Center, Inc. ("CCC") on behalf of the Rightsholder identified in the order details below. The license consists of the order details, the Marketplace Permissions General Terms and Conditions below, and any Rightsholder Terms and Conditions which are included below.

All payments must be made in full to CCC in accordance with the Marketplace Permissions General Terms and Conditions below.

Order Date	26-Jul-2023	Type of Use	Republish in a
Order License ID	1379428-1		thesis/dissertation
ISSN	1545-2123	Publisher Portion	ANNUAL REVIEWS
			Image/photo/illustration

LICENSED CONTENT

Publication Title	Annual review of plant biology	Publication Type	e-Journal
Article Title	Seasonal control of tuberization in potato: conserved elements with the flowering response.	Start Page	151
		End Page	180
		Issue	1
		Volume	57
Author/Editor	Annual Reviews, Inc.	URL	http://arjournals.annualreviews.org/loi/arplant.1
Date	01/01/2002		
Language	English		
Country	United States of America		
Rightsholder	Annual Reviews, Inc.		

REQUEST DETAILS

Portion Type	Image/photo/illustration	Distribution	Worldwide
Number of Images / Photos / Illustrations	1	Translation	Original language of publication
Format (select all that apply)	Print, Electronic	Copies for the Disabled?	No
Who Will Republish the Content?	Academic institution	Minor Editing Privileges?	Yes
Duration of Use	Life of current edition	Incidental Promotional Use?	No
Lifetime Unit Quantity	Up to 499	Currency	USD
Rights Requested	Main product		

NEW WORK DETAILS

Title	Investigating the functions of small non-coding RNAs in stolon-to-tuber transition stages of potato development	Institution Name	IISER Pune
		Expected Presentation Date	2023-08-08
Instructor Name	Prof. Anjan K. Banerjee		

ADDITIONAL DETAILS

Order Reference Number	N/A	The Requesting Person/Organization to Appear on the License	Nilam Malankar
------------------------	-----	---	----------------

REQUESTED CONTENT DETAILS

Title, Description or Numeric Reference of the Portion(s)	Figure 1	Title of the Article/Chapter the Portion Is From	Seasonal control of tuberization in potato: conserved elements with the flowering response.
Editor of Portion(s)	Rodríguez-Falcón, Mariana; Bou, Jordi; Prat, Salomé	Author of Portion(s)	Rodríguez-Falcón, Mariana; Bou, Jordi; Prat, Salomé
Volume / Edition	57		
Page or Page Range of Portion	151-180	Issue, if Republishing an Article From a Serial	1
		Publication Date of Portion	2006-06-02

Marketplace Permissions General Terms and Conditions

The following terms and conditions (“General Terms”), together with any applicable Publisher Terms and Conditions, govern User’s use of Works pursuant to the Licenses granted by Copyright Clearance Center, Inc. (“CCC”) on behalf of the applicable Rightsholders of such Works through CCC’s applicable Marketplace transactional licensing services (each, a “Service”).

- 1) **Definitions.** For purposes of these General Terms, the following definitions apply:
- “License” is the licensed use the User obtains via the Marketplace platform in a particular licensing transaction, as set forth in the Order Confirmation.
- “Order Confirmation” is the confirmation CCC provides to the User at the conclusion of each Marketplace transaction. “Order Confirmation Terms” are additional terms set forth on specific Order Confirmations not set forth in the General Terms that can include terms applicable to a particular CCC transactional licensing service and/or any Rightsholder-specific terms.
- “Rightsholder(s)” are the holders of copyright rights in the Works for which a User obtains licenses via the Marketplace platform, which are displayed on specific Order Confirmations.
- “Terms” means the terms and conditions set forth in these General Terms and any additional Order Confirmation Terms collectively.
- “User” or “you” is the person or entity making the use granted under the relevant License. Where the person accepting the Terms on behalf of a User is a freelancer or other third party who the User authorized to accept the General Terms on the User’s behalf, such person shall be deemed jointly a User for purposes of such Terms.
- “Work(s)” are the copyright protected works described in relevant Order Confirmations.

2) **Description of Service.** CCC’s Marketplace enables Users to obtain Licenses to use one or more Works in accordance with all relevant Terms. CCC grants Licenses as an agent on behalf of the copyright rightsholder identified in the relevant Order Confirmation.

3) **Applicability of Terms.** The Terms govern User’s use of Works in connection with the relevant License. In the event of any conflict between General Terms and Order Confirmation Terms, the latter shall govern. User acknowledges that Rightsholders have complete discretion whether to grant any permission, and whether to place any limitations on any grant, and that CCC has no right to supersede or to modify any such discretionary act by a Rightsholder.

4) **Representations; Acceptance.** By using the Service, User represents and warrants that User has been duly authorized by the User to accept, and hereby does accept, all Terms.

5) **Scope of License; Limitations and Obligations.** All Works and all rights therein, including copyright rights, remain the sole and exclusive property of the Rightsholder. The License provides only those rights expressly set forth in the terms and conveys no other rights in any Works

6) **General Payment Terms.** User may pay at time of checkout by credit card or choose to be invoiced. If the User chooses to be invoiced, the User shall: (i) remit payments in the manner identified on specific invoices, (ii) unless otherwise specifically stated in an Order Confirmation or separate written agreement, Users shall remit payments upon receipt of the relevant invoice from CCC, either by delivery or notification of availability of the invoice via the Marketplace platform, and (iii) if the User does not pay the invoice within 30 days of receipt, the User may incur a service charge of 1.5% per month or the maximum rate allowed by applicable law, whichever is less. While User may exercise the rights in



The phased short-interfering RNA siRD29(-) regulates GIBBERELLIN 3-OXIDASE 3 during stolon-to-tuber transitions in potato

Nilam N. Malankar ,¹ Kirtikumar R. Kondhare ,^{1,2} Kishan Saha ,¹ Mohit Mantri ¹ and Anjan K. Banerjee ^{1,*}

¹ Biology Division, Indian Institute of Science Education and Research (IISER Pune), Pune, 411008 Maharashtra, India
² Biochemical Sciences Division, CSIR - National Chemical Laboratory (CSIR-NCL), Pune, 411008 Maharashtra, India

*Author for correspondence: akb@iiserpune.ac.in

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (<https://academic.oup.com/plphys/pages/General-Instructions>) is Anjan K. Banerjee.

Abstract

Phased short-interfering RNAs (phasiRNAs) fine tune various stages of growth, development, and stress responses in plants. Potato (*Solanum tuberosum*) tuberization is a complex process, wherein a belowground modified stem (stolon) passes through developmental stages like swollen stolon and minituber before it matures to a potato. Previously, we identified several phasiRNA-producing loci (PHAS) from stolon-to-tuber transition stages. However, whether phasiRNAs mediate tuber development remains unknown. Here, we show that a gene encoding NB-ARC DOMAIN-CONTAINING DISEASE RESISTANCE PROTEIN (*StRGA4*; a PHAS locus) is targeted by *Stu*-microRNA482c to generate phasiRNAs. Interestingly, we observed that one of the phasiRNAs, referred as short-interfering RNA D29(-), i.e. siRD29(-), targets the gibberellin (GA) biosynthesis gene *GIBBERELLIN 3-OXIDASE 3* (*StGA3ox3*). Since regulation of bioactive GA levels in stolons controls tuber development, we hypothesized that a gene regulatory module, *Stu*-miR482c-*StRGA4*-siRD29(-)-*StGA3ox3*, could govern tuber development. Through transient expression assays and small RNA sequencing, generation of siRD29(-) and its phase was confirmed in planta. Notably, the expression of *StGA3ox3* was higher in swollen stolon compared to stolon, whereas siRD29(-) showed a negative association with *StGA3ox3* expression. Antisense (AS) lines of *StGA3ox3* produced more tubers compared to wild type. As expected, *StRGA4* overexpression (OE) lines had high levels of siRD29(-) and mimicked the phenotypes of *StGA3ox3*-AS lines, indicating the functionality of this module in potato. *In vitro* tuberization assays (with or without a GA inhibitor) using *StGA3ox3* antisense lines and overexpression lines of *StGA3ox3* or *StRGA4* revealed that *StGA3ox3* controls the tuber stalk development. Taken together, our findings suggest that a phasiRNA, siRD29(-), mediates the regulation of *StGA3ox3* during stolon-to-tuber transitions in potato.

Introduction

Small noncoding RNAs (sRNAs) control various aspects of plant growth and development (Liu et al. 2020; Dong et al. 2022). MicroRNAs (miRNAs) and phased short-interfering RNAs (phasiRNAs) constitute a major category of sRNAs and regulate the expression of target genes via transcriptional or posttranscriptional gene silencing (Fei et al. 2013).

miRNAs are 21- to 24-nt sRNAs, which form a RNA-induced silencing complex (RISC) with ARGONAUTE 1 (AGO1) to target mRNAs (Rogers and Chen 2013; Borges and Martienssen 2015). The AGO1-RISC complex loaded with 22-nt miRNAs can trigger the biogenesis of sRNAs from target mRNAs. With the help of SUPPRESSOR OF GENE SILENCING3 (SGS3) and RNA-DEPENDENT RNA POLYMERASE6 (RDR6), cleaved mRNAs form dsRNAs

(Yoshikawa et al. 2005). The resulting dsRNAs are then cleaved by a DICER-LIKE 4 protein (DCL4), forming phasiRNA duplexes that are distinctive and generated in phased configuration (Allen et al. 2005; Chen et al. 2010). The loci generating phased siRNAs are called PHAS loci.

Although numerous PHAS loci have been identified from diverse plant species, only selective phasiRNAs are characterized for their functions in plant development. A subset of phasiRNAs, known as trans-acting siRNAs (tasiRNAs), is involved in seed germination, leaf and root development, shoot apical meristem (SAM) initiation, flowering time, and pre-meiotic reproductive development (Liu et al. 2020). Major trans-acting siRNA producing loci (TAS) members are conserved in plants. For example, miRNA390-TAS3-mediated regulation of the ARF (AUXIN RESPONSE FACTOR) pathway is conserved across the plant lineages and controls leaf morphogenesis, developmental timing and patterning, lateral root growth, and somatic embryogenesis (Xia et al. 2017). miR166 and tasiRNAs targeting ARFs regulate shoot meristem initiation and the maintenance of leaf polarity in maize (*Zea mays*) and rice (*Oryza sativa*) (Nagasaki et al. 2007; Nogueira et al. 2007). Another relatively conserved phasiRNA pathway is miR828 targeting TAS4 or MYB transcription factor (TF) genes (Xia et al. 2012) that are involved in biosynthesis of anthocyanin, flavonoids, lignin (Bonar et al. 2018; Tirumalai et al. 2019), and fruit development (Rock 2013). Nonconserved phasiRNA pathways, such as miR3954-siRNAs-NAC TFs, affect flowering time in citrus (*Citrus sinensis*) (Liu et al. 2017; Ma et al. 2018). A recent study showed that miR9678, a wheat- (*Triticum aestivum*) specific miRNA, targets the long noncoding RNA, *Wheat Seed Germination Associated RNA* (WSGAR), to produce phasiRNAs, which regulate the germination process via the altered response of 2 phytohormones, abscisic acid, and gibberellin (GA) (Guo et al. 2018). However, siRNA targets in this pathway are unknown.

Tuber development mechanism (tuberization) in potato (*Solanum tuberosum*) is a complex process governed by a coordinated action of various environmental, biochemical, and genetic factors (Hannapel et al. 2017; Kondhare et al. 2020). During tuberization, a belowground modified stem (stolon) passes through different developmental transitions starting from swelling at the subapical region to the development of a minituber, before maturing to a potato. At the onset of tuber formation, a dynamic change occurs in GA and auxin metabolism and their signaling pathways in the stolon. Several genes associated with auxin biosynthesis (YUCCA family of FLAVIN-CONTAINING MONOOXYGENASE; StYUC-LIKE1), transport (PIN-FORMED proteins; StPIN2 and -4), and signaling pathways (AUXIN RESPONSE FACTOR; ARF and AUXIN/INDOLE-3-ACETIC ACID PROTEINS; AUX/IAA proteins) show differential expression during stolon-to-tuber transition stages (Kloosterman et al. 2008; Roumeliotis et al. 2012b; 2013b). Moreover, the expression of GA catabolism gene GIBBERELLIN 2-OXIDASE 1 (StGA2ox1) increases (Kloosterman et al. 2007), whereas the expression of GA biosynthesis genes—GIBBERELLIN 20-OXIDASE 1

(StGA20ox1) and GIBBERELLIN 3-OXIDASE 2 (StGA3ox2)—decreases under tuber-inducing short-day conditions (Carrera et al. 2000; Bou-torrent et al. 2011). As a result, auxin levels increase at the stolon tip, which is accompanied by the reduction of GA levels in the subapical part of the stolon (Roumeliotis et al. 2012a; 2012b). This ceases elongation of the stolon and initiates swelling at the subapical region, followed by accumulation of starch and storage proteins to favor tuber maturation. Hence, the spatiotemporal changes in expression profiles of auxin and GA metabolism genes are crucial for early stages of stolon-to-tuber transitions. The changes in GA levels are controlled by regulation of StGA20ox1 and StGA2ox1 through StBEL5 (BEL1-LIKE TF 5) and POTH1 (POTATO HOMEODOMAIN 1) homeodomain TFs as well as StSP6A protein (a FLOWERING LOCUS T ortholog in potato). These factors act as positive regulators of tuberization (Banerjee et al. 2006; Navarro et al. 2011; Mahajan et al. 2012). Specifically, the StBEL5-POTH1 heterodimer binds to the promoter sequences of StGA20ox1 and negatively regulates its expression (Chen et al. 2004). This interaction occurs through tandem TGAC core motifs present in the promoter sequences of the target genes. Overexpression of StBEL5 and StSP6A independently resulted in an increased levels of StGA2ox1 in stolons. The promoter of StGA2ox1 showed the presence of 2 tandem TGAC motifs and StBEL5-POTH1 has been shown to bind to this sequence and induce gene expression (Lin et al. 2013; Sharma et al. 2016).

Zhang et al. (2020) have identified reproductive phasiRNAs that are essential to silence genes associated with early events of meiosis in rice. Previously, we have identified several phasiRNAs that are potentially involved in regulating key tuberization genes in potato (Kondhare et al. 2018; 2021). Specific phasiRNAs were predicted to cleave GA metabolism and auxin signaling genes. In the present study, a well-conserved miR482 family member, Stu-miR482c, was predicted to cleave the transcript of a gene encoding NB-ARC DOMAIN-CONTAINING DISEASE RESISTANCE PROTEIN (StRGA4) to generate phasiRNAs. Further, 1 of the phasiRNAs, referred as siRD29(-), could target the transcript of the GA biosynthesis gene (StGA3ox3). The function of this StGA3ox3 isoform is unknown in potato. To investigate the existence and functionality of the Stu-miR482c-StRGA4-siRD29(-)-StGA3ox3 gene regulatory module, we asked following questions: (i) Does siRD29(-) generate from the Stu-miR482c-cleaved StRGA4 locus? (ii) Is this siRNA generated during stolon-to-tuber transition stages? (iii) Does siRD29(-) target StGA3ox3 in a tissue-specific manner? (iv) What is the role of StGA3ox3 in potato development? (v) Does this small RNA target gene pair (siRD29[-]-StGA3ox3) mediate the stolon-to-tuber transitions?

Here, we have validated the generation of siRD29(-) from its PHAS locus (StRGA4) and confirmed StGA3ox3 as its target. Further, we showed that the Stu-miR482c-StRGA4-siRD29(-)-StGA3ox3 gene regulatory module is functional during stolon-to-tuber transitions and the StGA3ox3 isoform is potentially involved in GA₁ and GA₃ biosynthesis. Overall,

our study revealed phasiRNA-mediated regulation of a GA biosynthesis gene during tuber development in potato.

Results

MicroRNA482c cleaves StRGA4, a PHAS locus encoding Nb-ARC DOMAIN-CONTAINING DISEASE RESISTANCE PROTEIN in potato

Previously, we identified 830 putative phasiRNA producing (PHAS) loci from sRNA-sequencing data sets of stolon-to-tuber transition stages in potato (Kondhare et al. 2018). Among them, we found that a PHAS locus (Chr09:46314301..46314552) can generate a phased siRNA (phasiRNA), which targets a gibberellin biosynthesis gene *StGA3ox* (Soltu.DM.01G018850.1). This PHAS locus was located in the exonic region of a NB-ARC DOMAIN-CONTAINING DISEASE RESISTANCE PROTEIN—*StRGA4* (Soltu.DM.09G019040.1)—and predicted to be cleaved by *Stu-miR482c* to generate phasiRNAs (Supplemental Table S1; Fig. 1A). Through a modified 5' RLM-RACE, we confirmed that *Stu-miR482c* cleaves *StRGA4* transcript with a high frequency (8 of 8) at the predicted site (Fig. 1B). Based on the cleavage site, the phase of phasiRNA that targets *StGA3ox* was mapped on the *StRGA4* transcript at the 29th position on the antisense strand, hence referred as siRD29(-) (Fig. 1A).

SiRD29(-) generation is RDR6 dependent

To test if microRNA482c (*Stu-miR482c*) is responsible for generating siRD29(-) from *StRGA4* transcript, we coinfiltrated *Stu-miR482c* and *StRGA4* OE constructs in leaves of *Nicotiana benthamiana* plants. Dot blot analysis indicated the accumulation of siRD29(-) in the leaves following 48 hrs of coinfiltration (Fig. 1C). Further, we sequence verified this siRNA (Supplemental Fig. S1). No siRNAs were generated in the empty vector-infiltrated leaf samples (control). RDR6 is an essential factor in conventional biogenesis of phasiRNAs (Yoshikawa et al. 2005). To investigate if siRD29(-) is generated in a RDR6-dependent manner, coinfiltration experiment was also performed in the *RDR6-RNAi* line of *N. benthamiana*. These plants have a significantly reduced *RDR6* transcript level and thus have low levels of phasiRNAs (Schwach et al. 2005). Our analysis indicated that the generation of siRD29(-) was reduced by 96% in the coinfiltrated leaves of *RDR6i* plants compared to wild type (WT) (Fig. 1D). These results confirmed that siRD29(-) is generated from *StRGA4* transcript in RDR6-dependent manner post cleavage by *Stu-miR482c*.

A phasiRNA siRD29(-) targets StGA3ox3

The phylogenetic analysis revealed a close conservation of this isoform with *Arabidopsis* (*Arabidopsis thaliana*) *AtGA3ox3* (NP_193900.1) (Supplemental Fig. S2). Thus, we referred this gene as *StGA3ox3* henceforth. In silico analysis predicted that siRD29(-) targets *StGA3ox3* with an expectancy value of 2.5 ($E = 2.5$). Using a modified 5' RLM-RACE,

we confirmed the cleavage of the *StGA3ox3* transcript by siRD29(-) with a high frequency (9 of 9) at 11th/12th nucleotide position (Fig. 1E). For in planta validation of siRD29(-) targeting *StGA3ox3*, a transient coexpression of 35S::*StGA3ox3*, 35S::miR482c and 35S::*StRGA4* was carried out in leaves of WT and *RDR6i* plants of *N. benthamiana*. The transcript levels of *StGA3ox3* in coinfiltrated samples were significantly reduced compared to control (Fig. 1F). As expected, coinfiltrated *RDR6i* lines did not show any change in *StGA3ox3* levels due to low levels of siRD29(-) in these plants (Fig. 1G). Altogether, these results suggest that siRD29(-) acts in a *trans*-manner to regulate *StGA3ox3*.

Further, RT-qPCR analysis revealed that siRD29(-) was highly expressed in leaves, followed by node, shoot apex, stolon, and stem under LD condition (Fig. 1H). During stolon-to-tuber transition stages, it showed highest expression in SD-induced stolons (Fig. 1I).

Stu-miR482c triggers secondary siRNAs from StRGA4 locus in planta

To confirm the generation of secondary siRNAs from *StRGA4* post *Stu-miR482c* cleavage in planta, we performed small RNA sequencing of *N. benthamiana* leaves coinfiltrated with *Stu-miR482c* and *StRGA4* OE constructs. sRNAs were isolated from the infiltrated leaves (48 hrs post infiltration) and subjected for deep sequencing. The size distribution profile of sRNAs indicated that *StRGA4* expression did not have any effect on sRNA population from *N. benthamiana*, but an increase in the proportion of 21 nt small RNAs was observed in coinfiltrated samples as compared to *StRGA4*-infiltrated samples (Fig. 2A). Further, we mapped sRNA reads to the *StRGA4* transcript. *StRGA4*-specific sRNA reads were absent in vector control samples but were abundant in coinfiltrated samples in the presence of *Stu-miR482c* (Fig. 2B). The secondary siRNA phase began at the 1st nucleotide from the *Stu-miR482c* cleavage site, which extended up to 29th position, where siRD29(-) is located (Fig. 2C). Subsequently, the number of reads for siRNAs decreased (Supplemental Table S2). The first 3 phasiRNAs (D1-D3) from the *StRGA4* locus had the highest number of sRNA reads (Supplemental Table S2). Though, the position of siRD29(-) was far from the *Stu-miR482c* cleavage site, we still obtained a higher number of sRNA reads in comparison with other siRNAs near the cleavage site (Fig. 2C). Overall, high-throughput sequencing of coinfiltrated samples confirmed that siRD29(-) is generated post *Stu-miR482c* cleavage at the 29th phase from the *StRGA4* PHAS locus.

StGA3ox3 is highly expressed in the swollen stolon under SD

StGA3ox3 showed a ubiquitous expression in all tissue types tested, with the highest expression in leaves, followed by node, shoot apex, and stem tissues (Fig. 3A). The effect of SD photoperiod was seen to be gradual as the leaves from WT plants did not show a significant reduction in *StGA3ox3* levels

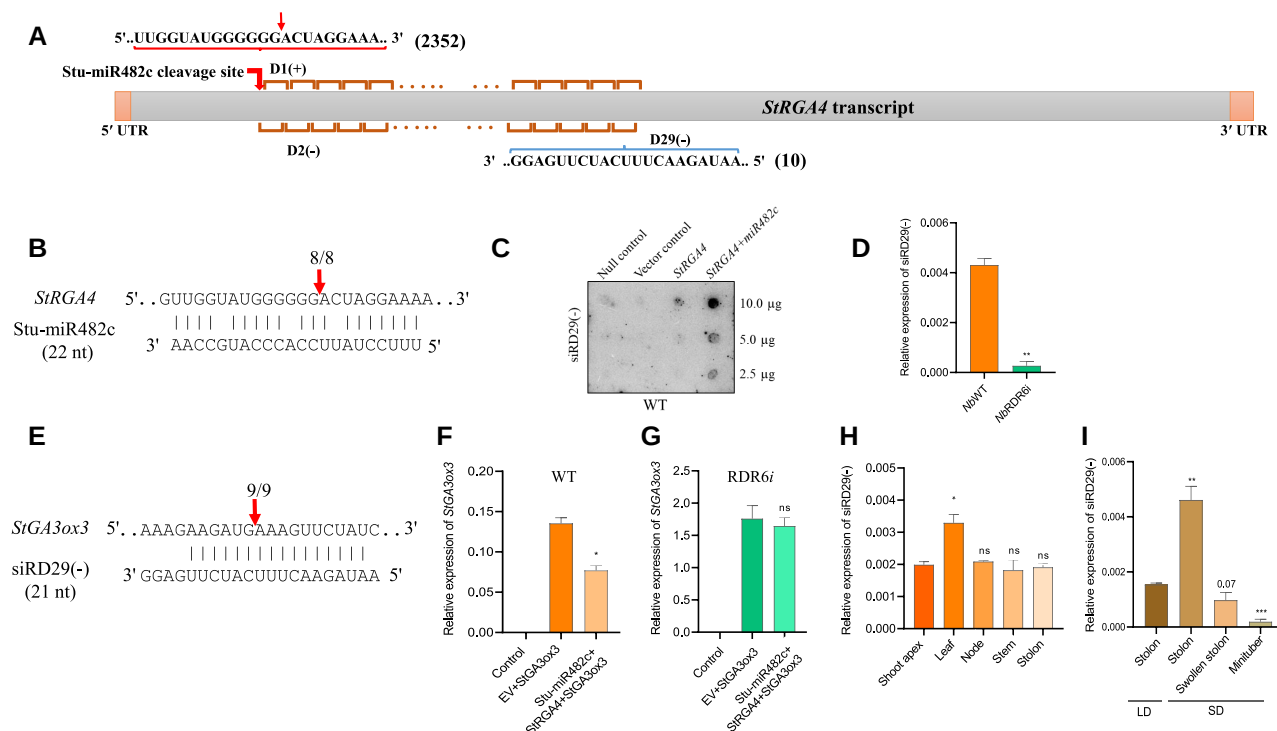


Figure 1. A phasiRNA *siRD29(-)* generated from *StGA4* transcript, which targets *StGA3ox3* mRNA. **A**) A schematic representation of the *StGA4* transcript and *Stu-miR482c* cleavage site on *StGA4* mRNA. Putative phasiRNAs and the location of a phasiRNA *siRD29(-)* on the antisense strand are also shown. The number in parentheses indicates the read count obtained in small RNA sequencing dataset from stolons (Kondhare et al. 2018). **B**) Modified 5' RLM-RACE results depicting the cleavage site mapping of *Stu-miR482c* on *StGA4* transcript. Arrow shows the site of cleavage. The numbers (8/8) represent the frequency of clones with the same cleavage site. **C**) Dot blot-based detection of a phasiRNA *siRD29(-)* in leaves of wild-type *N. benthamiana* (NbWT) plants following coinfiltration of gene constructs using *A. tumefaciens*. The increased abundance of *siRD29(-)* was detected using the blot paper. Null control, mock infiltrated leaves; vector control, empty vector-infiltrated leaves; *StGA4*, leaves infiltrated with 35S::*StGA4* construct; *StGA4* + *miR482c*, leaves coinfiltrated with 35S::*StGA4* and 35S::pre-*miR482c* gene constructs in a binary vector. **D**) Quantification of *siRD29(-)* levels from agroinfiltrated samples of WT plant and *RDR6i* line of *N. benthamiana* with 35S::*StGA4* and 35S::pre-*miR482c* gene constructs. *NtU6* was used as reference gene. **E**) Modified 5' RLM-RACE results representing the cleavage site mapping of a phasiRNA *siRD29(-)* on *StGA3ox3* transcript. Arrow shows the site of cleavage. The numbers (9/9) represent the frequency of clones showing the same cleavage site. **F**) *StGA3ox3* mRNA accumulation 48 h after coinfiltration in WT *N. benthamiana* leaves. Control, mock infiltrated leaves; EV + *StGA3ox3*, leaves coinfiltrated with empty vector and 35S::*StGA3ox3* construct; *Stu-miR482c* + *StGA4* + *StGA3ox3*, leaves coinfiltrated with 35S::pre-*miR482c*, 35S::*StGA4* and 35S::*StGA3ox3* gene constructs. The transcript abundance was plotted as the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001). **G**) *StGA3ox3* mRNA accumulation 48 h after coinfiltration in *RDR6i* *N. benthamiana* leaves. **H**) Expression of *siRD29(-)* from the 2-wk-old potato plant. *StU6* was used as reference gene. Different tissue types (shoot apex, leaf, stem, node, and stolon) were used for the analysis. The abundance of *siRD29(-)* was plotted using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001). **I**) The levels of *siRD29(-)* during stolon-to-tuber transition stages. For D and F to I, error bars indicate standard error of means (SEM) from 3 biological replicates ($n = 3$). Asterisks (1, 2, and 3) indicate significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.005$ respectively, using 1-way ANOVA, followed by the Tukey HSD test and Student's *t*-test. ns, not significant at $P < 0.05$; LD, long day; SD, short day.

after 4 days of SD induction, but it was reduced 7 days post SD induction (Fig. 3B). Further, at 14 days of SD, *StGA3ox3* expression significantly reduced in leaves, petioles, and nodes, but it was high in the shoot apex compared to LD conditions (Fig. 3C). The expression of *StGA3ox3* remains unchanged in root and stem tissues under LD/SD conditions (Fig. 3C). It was evident that *StGA3ox3* was highly expressed in swollen stolons, but its expression declined in minitubers (Fig. 3D). However, the expression of other known GA metabolism genes (*StGA3ox2*, *StGA20ox2*, and *StGA20ox1*) and tuberization activator *StBEL5* during stolon-to-tuber transition stages were in line with the previous reports (Supplemental Fig. S3).

The pattern of GUS activity using 5-wk-old in vitro plants of *promStGA3ox3::GUS-pBI121* lines matched with *StGA3ox3* gene expression (Fig. 3E). GUS expression was observed in leaves, roots, and the junction of stem petiole and stem roots (Fig. 3, E, F, I and L). In the leaf, GUS expression was prominently observed in the guard and mesophyll cells (Fig. 3, G and H), respectively. Longitudinal (Fig. 3J) and transverse (Fig. 3K) sections of the stem petiole junction revealed that the *StGA3ox3* promoter expresses in the cortex cells and not in the meristem region. Transverse section of roots indicated GUS expression in the cortex region. A faint GUS expression was also seen in the phloem region (Fig. 3M). Strong GUS

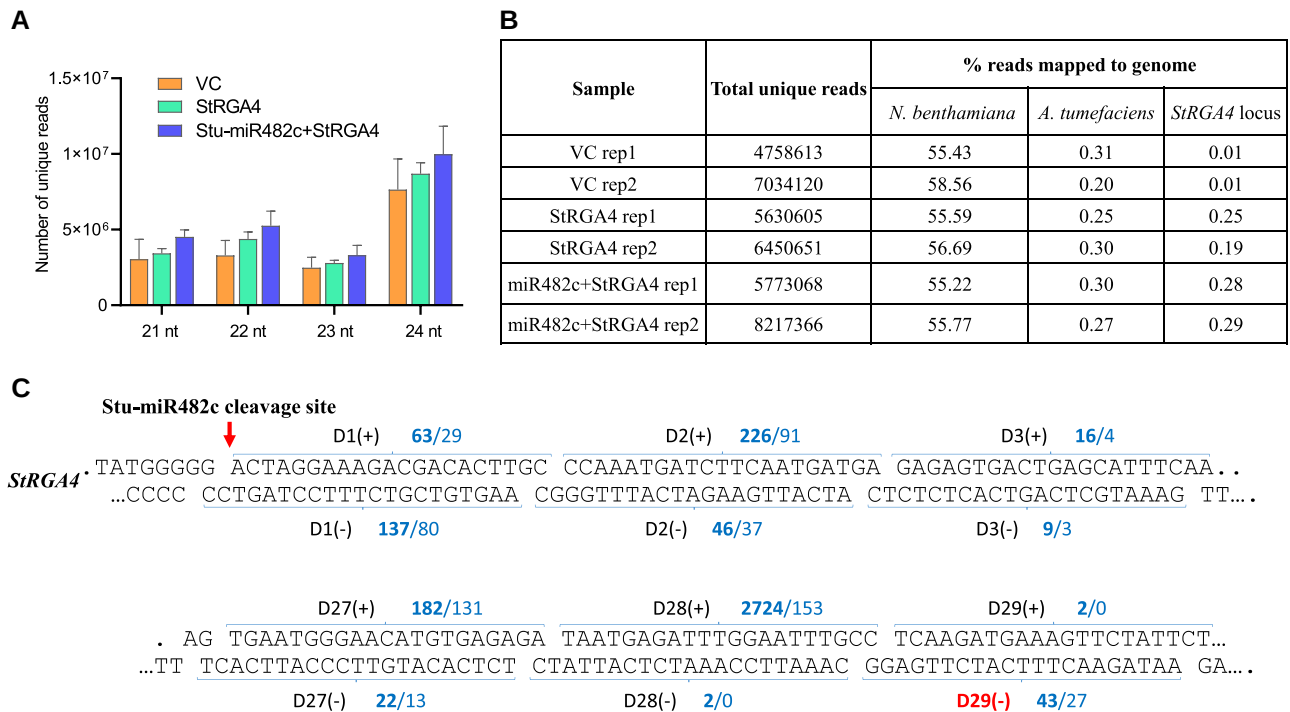


Figure 2. A phasiRNA, siRD29(–), is generated from the antisense strand of *StRGA4* transcript at the 29th phase post Stu-miR482c-mediated cleavage. **A**) Abundance of small RNAs (21 to 24 nt) in coinfiltrated leaf samples of *N. benthamiana*. VC, leaves infiltrated with empty vector; *StRGA4*, leaves infiltrated with 35S::*StRGA4*; *StRGA4* + Stu-miR482c, leaves infiltrated with 35S::*StRGA4* and 35S::pre-miR482c constructs. Error bars indicate standard deviation of means (SD) from 2 biological replicates ($n = 2$). **B**) Total read counts from small RNA sequencing of infiltrated samples of *N. benthamiana* for control, *StRGA4*, and coinfiltration of 35S::*StRGA4* and 35S::pre-miR482c gene constructs. The mapping percentage of unique reads to the genome of *N. benthamiana*, *A. tumefaciens*, and *StRGA4* locus is also shown. **C**) Phased siRNAs register with Stu-miR482c-guided cleavage site. Phased siRNAs (21 nt) are marked with the position from the cleavage site. The numbers above and below indicated the reads obtained from the leaf samples coinfiltrated with *StRGA4* and pre-miR482c gene constructs versus those infiltrated with only *StRGA4* gene construct. For a simplified view, phasiRNAs generated from the first 3 phases (D1–3) and those from 27th to 29th positions (D27–29) are represented.

activity was observed in swollen stolons (Fig. 3O), whereas a faint GUS expression was noted in minitubers and elongated stolons (Fig. 3, N and P, respectively). Similarly, GUS and StGA3ox3 transcript expression was increased in swollen stolons and was reduced in tubers (Fig. 3Q; Supplemental Fig. 4A). We noticed that GUS protein activity had not changed in swollen stolons but reduced at the minituber stage (Supplemental Fig. 54B). Since, the total protein content increases during stolon-to-tuber stages (Supplemental Fig. 54C), the effect of induced GUS expression did not match with the GUS protein activity in swollen stolons.

Altered expression of StGA3ox3 affects leaf size and chlorophyll content with no effect on tuber weight

To characterize the function of StGA3ox3 in potato, 5 constitutive StGA3ox3-OE lines in andigena were generated (Supplemental Fig. 55A). Of them, 3 independent lines (referred as #OE2, #OE3, and #OE5) with moderate or high levels of StGA3ox3 expression were chosen for further analysis (Fig. 4, A and B). Leaflet numbers were the same but the overall leaf size was larger and the terminal leaflet length was significantly higher in OE lines than WT (Fig. 4,

C and D). However, no change in the total leaf length was noted (Supplemental Fig. S6B). OE lines showed an increase in plant height compared to WT (Fig. 4E). The chlorophyll content of the leaves was reduced in StGA3ox3-OE lines (Fig. 4F), whereas #OE3 had a reduced anthocyanin content compared to WT (Fig. 4G).

To assess the effect of StGA3ox3 knockdown, we generated its antisense (AS) lines in andigena (Supplemental Fig. S5B). We selected 2 antisense lines (referred as #AS1 and #AS3) with more than 80% reduction in the StGA3ox3 transcript levels (Fig. 4, A and B). StGA3ox3-AS lines showed a reduction in plant height accompanied by a significant decrease in the internodal distance (Fig. 4E, Supplemental Fig. S6A). Although there was no change in the length of terminal leaflet as observed in StGA3ox3-OE lines (Supplemental Fig. S6C), the leaflets of the StGA3ox3-AS lines were flattened compared to WT (Fig. 4C). Evaluation of the tuberization potential of OE and AS lines of StGA3ox3 showed that the overall tuber weight remains unchanged in both transgenic lines (Supplemental Fig. S6, D to F). However, AS lines produced significantly higher tuber numbers per plant (Fig. 4, H and I; Supplemental Fig. S6, H and I). Grafting experiment was carried out to test if leaf morphology or other pleiotropic

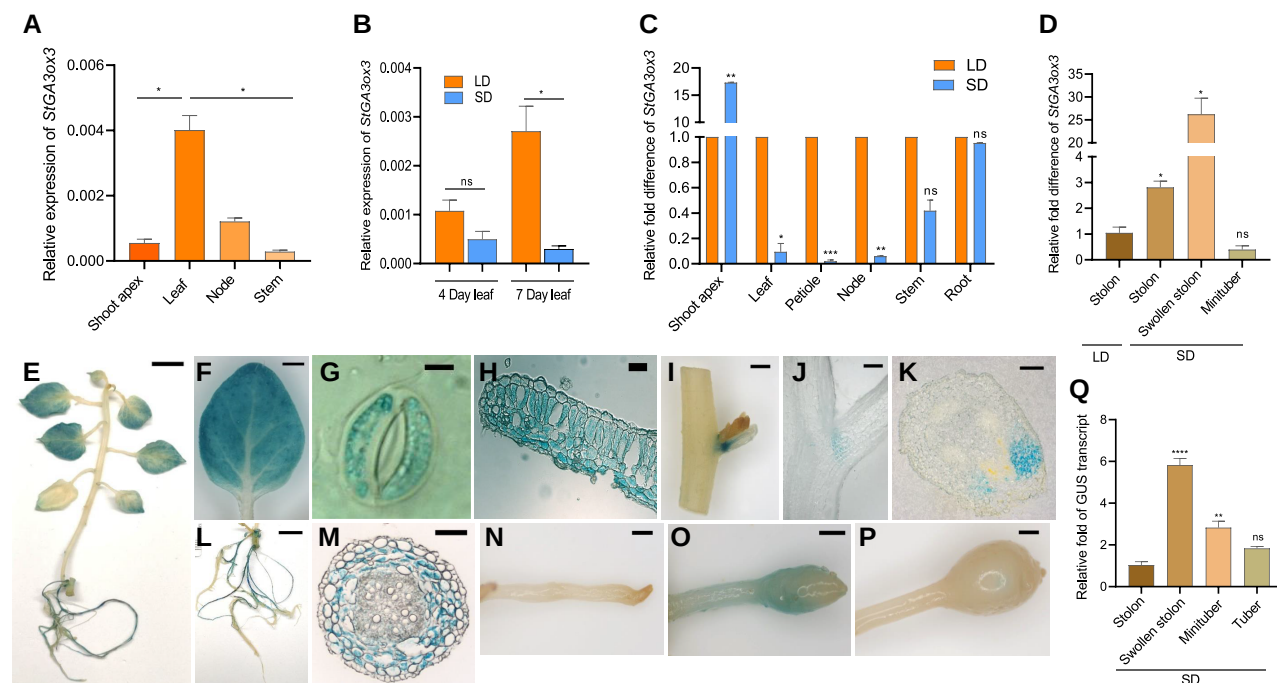


Figure 3. *StGA3ox3* exhibits tissue-specific gene expression pattern, and short day induces its expression in swollen stolon. **A)** Accumulation of *StGA3ox3* transcript in various tissues of WT andigena potato plants. Four tissue types of 2-wk-old soil-grown potato plants, such as shoot apex, leaf, node, and stem were used for analysis. *StEIF3e* was used as a reference gene. Statistical significance was calculated in comparison with the leaf transcript abundance. **B)** Effect of short-day (SD) and long-day (LD) photoperiods (4 and 7 days) on the expression of *StGA3ox3* in leaves of potato plants. **C)** Effect of photoperiod on the expression of *StGA3ox3* in different tissue types of potato plants, subjected to LD or SD induction for 14 days, following 8 wk in soil under LD conditions. Fold change of the gene expression in each tissue type for SD treatment is compared to respective LD expression. **D)** Gene expression of *StGA3ox3* during stolon-to-tuber transition stages (stolon, swollen stolon, and minituber) harvested from potato plants following 10 days of SD or LD induction. Fold-change of the gene expression in SD is compared to LD for each tissue type. For B to C, error bars indicate standard error of means (SEM) from 3 biological replicates ($n = 3$). Asterisks (1, 2, and 3) indicate significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively, using Student's *t*-test. ns, not significant at $P > 0.05$. **E to P)** GUS activity in *promStGA3ox3::GUS* transgenic lines. GUS activity in 5-wk-old in vitro-grown entire plant (**E**), leaf and guard cells (**F** and **G**), longitudinal section of leaf blade (**H**), petiole stem nodal junction (**I**), longitudinal and transverse sections of petiole stem nodal junction (**J** and **K**), roots and their transverse sections showing GUS expression in the cortex region (**L** and **M**), stolons, swollen stolons, and minitubers (**N**, **O** and **P**) harvested from 8-wk-old soil grown plants of *promStGA3ox3::GUS* induced with LD/SD conditions for 10 days. Faint GUS expression was observed in minitubers (**P**). Scale bars, **E** and **L** = 1 cm; **F**, **I**, **N**, **O**, and **P** = 1 mm; **J** = 500 μ m; **K** = 200 μ m; **M** = 100 μ m; and **G** and **H** = 20 μ m. **Q)** The GUS transcript levels during stolon-to-tuber transition stages harvested from *promStGA3ox3::GUS* transgenic lines following 10 days of SD induction. For **A**, **D**, and **Q**, error bars indicate standard error of means (SEM) from 3 biological replicates ($n = 3$). Asterisks (2 and 3) indicate significant differences at $P < 0.01$ and $P < 0.005$, respectively, using 1-way ANOVA, followed by a Tukey HSD test. ns, not significant at $P < 0.05$.

phenotypes of the transgenic lines have any effect on tuber numbers. We observed no changes in tuber numbers for OE/OE, WT/OE, and OE/WT grafts (Supplemental Fig. S7). The tuber numbers in heterografts of WT/AS and AS/WT were also comparable to WT/WT homografts, suggesting that an increase of the tuber numbers in *StGA3ox3*-AS lines might not be a result of the change in the leaf morphology. Instead, it could be due to the impaired bioactive GA levels.

StGA4 overexpression lines have high levels of siRD29(-) and mimic the tuber phenotype of *StGA3ox3*-AS lines

Disease resistance genes from the *Solanaceae* family are known to be targeted by miR482/2118 members to produce secondary phasiRNAs (Zhai et al. 2011; Shivaprasad et al.

2012) that further regulate numerous defense-related genes. In the absence of any pathogen attack, the miR482 family-mediated secondary phasiRNA cascade remains active. After pathogen attack, miR482 levels reduces in order to express and maintain high levels of disease resistance genes. We utilized the principle of this signaling cascade to overexpress siRD29(-) in potato. To enhance the expression of siRD29(-), constitutive OE lines of *StGA4* (OE8 and OE10) were generated in andigena plants. These lines exhibited reduced levels of *StGA4* (Fig. 5A) as the transcript is being targeted by increased levels of Stu-miR482c (Fig. 5B). Both OE lines showed 2- to 2.5-fold increase of siRD29(-) levels in the leaves (Fig. 5C). The corresponding ~70% decrease in *StGA3ox3* levels was observed (Fig. 5D). *StGA4*-OE lines mimicked the specific phenotypes of *StGA3ox3*-AS lines, for example, reduced plant height (Fig. 5E), no change in the final tuber

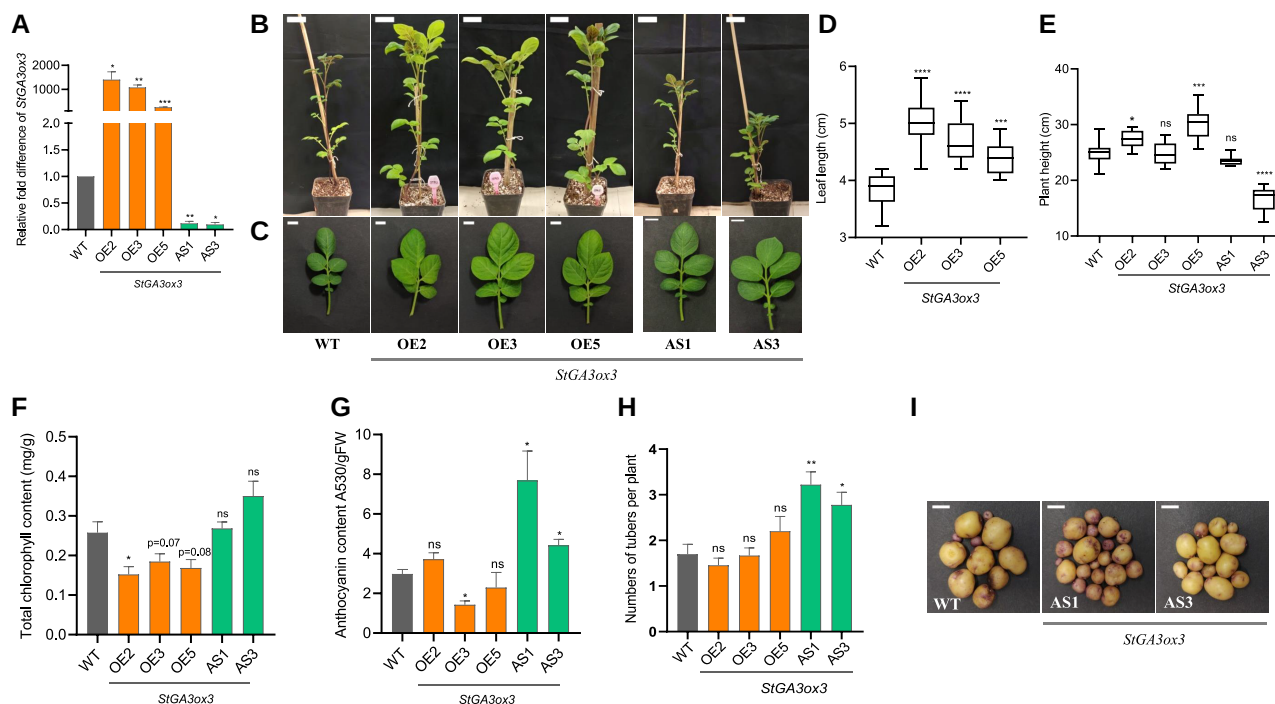


Figure 4. StGA3ox3 overexpression affects leaf size and chlorophyll content, whereas its knockdown alters the number of tubers in potato. **A)** Expression levels of StGA3ox3 in the 5th leaf of overexpression (OE2, OE3, and OE5) and antisense (AS1 and AS3) lines compared to WT. StEIF3e was used as a reference gene for RT-qPCR analysis. Error bars indicate standard error of means (SEM) from 3 biological replicates ($n = 3$). Asterisks (1, 2, and 3) indicate significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, using Student's t -test. ns, not significant at $P > 0.05$. **B)** Plant architecture of StGA3ox3-OE and -AS lines along with WT. Scale bar = 5 cm. **C)** Representative images of the 5th leaf from WT, StGA3ox3-OE, and StGA3ox3-AS lines. Scale bar = 1 cm. **D)** Terminal leaflet length of fully expanded 5th leaf in StGA3ox3-OE lines in comparison with WT. Data are from 12 independent plants for each line. **E)** Plant height in StGA3ox3-OE and -AS lines in comparison with WT. For D to E, each min to max whisker boxplot is prepared using data from 12 individual plants. Transverse lines show median of datapoints. **F)** Total chlorophyll content from the 5th leaf of WT, StGA3ox3-OE, and StGA3ox3-AS lines. **G)** Estimation of anthocyanin content in the 5th leaf from the shoot apex in StGA3ox3-OE and StGA3ox3-AS lines and WT plants. Panels F and G, $n = 3$. **H)** Number of tubers in StGA3ox3-OE and StGA3ox3-AS lines compared to WT. Data were taken from soil-grown plants after 1 mo of SD induction and plotted from 10 individual plants per line. Error bars indicate standard error of means (SEM). Asterisks (1, 2, 3, and 4) indicate significant differences at $P < 0.05$, $P < 0.01$, $P < 0.005$, and $P < 0.001$, respectively, using 1-way ANOVA, followed by Dunnett test. ns, not significant at $P < 0.05$. **I)** Representative images for the tubers obtained. Each image contains tubers from 6 plants per line. Scale bar = 1 cm.

weight (Supplemental Fig. S6G), but an increased tuber numbers (Fig. 5, F and G). We also quantified module-related genes in SD-induced stolons of StGA4-OE lines which exhibited increased levels of siRD29(-), followed by a reduction in the level of StGA3ox3 (Fig. 5H). StGA3ox3-OE lines (#OE3 and #OE5) showed induced expression of siRD29(-) in stolons possibly to balance the levels of StGA3ox3 (Supplemental Fig. S8A). StGA3ox3-AS lines also exhibited increased abundance of siRD29(-). However, other module components (StGA4 and Stu-miR482c) in these lines did not vary compared to WT (Supplemental Fig. S8B). These results suggest that the gene regulatory module (GRM) is functional during stolon-to-tuber transitions of potato.

StGA3ox3 controls tuber stalk development under *in vitro* conditions

A transient overexpression of StGA3ox3 in potato leaves led to an increase in GA₁ levels post 48 hrs of agroinfiltration,

suggesting that StGA3ox3 could contribute to the GA₁ biosynthesis pathway (Supplemental Fig. S9). Further, to study if StGA3ox3 influences the earliness for tuber formation, we conducted *in vitro* tuberization assays using OE lines of StGA3ox3 and StGA4 and StGA3ox3-AS lines. StGA3ox3-OE exhibited slight delay in tuberization compared to control (Supplemental Fig. S10A), whereas StGA3ox3-AS and StGA4-OE lines did not show delay in tuber formation. We observed sessile tuber formation in WT andigena plants. Interestingly, more than 50% tubers of StGA3ox3-OE lines showed stalk formation as compared to none in WT (Fig. 6A), possibly due to increased levels of bioactive GAs in the tuberizing nodes. Consistently, a transient overexpression of StGA3ox3 in potato leaves led to an increase in GA₁ levels following 48 hrs of agroinfiltration, suggesting that StGA3ox3 could contribute to the GA₁ biosynthesis pathway (Supplemental Fig. S9). StGA3ox3-AS and StGA4-OE lines exhibited sessile tubers similar to control plants (Fig. 6, A and B). Further, addition

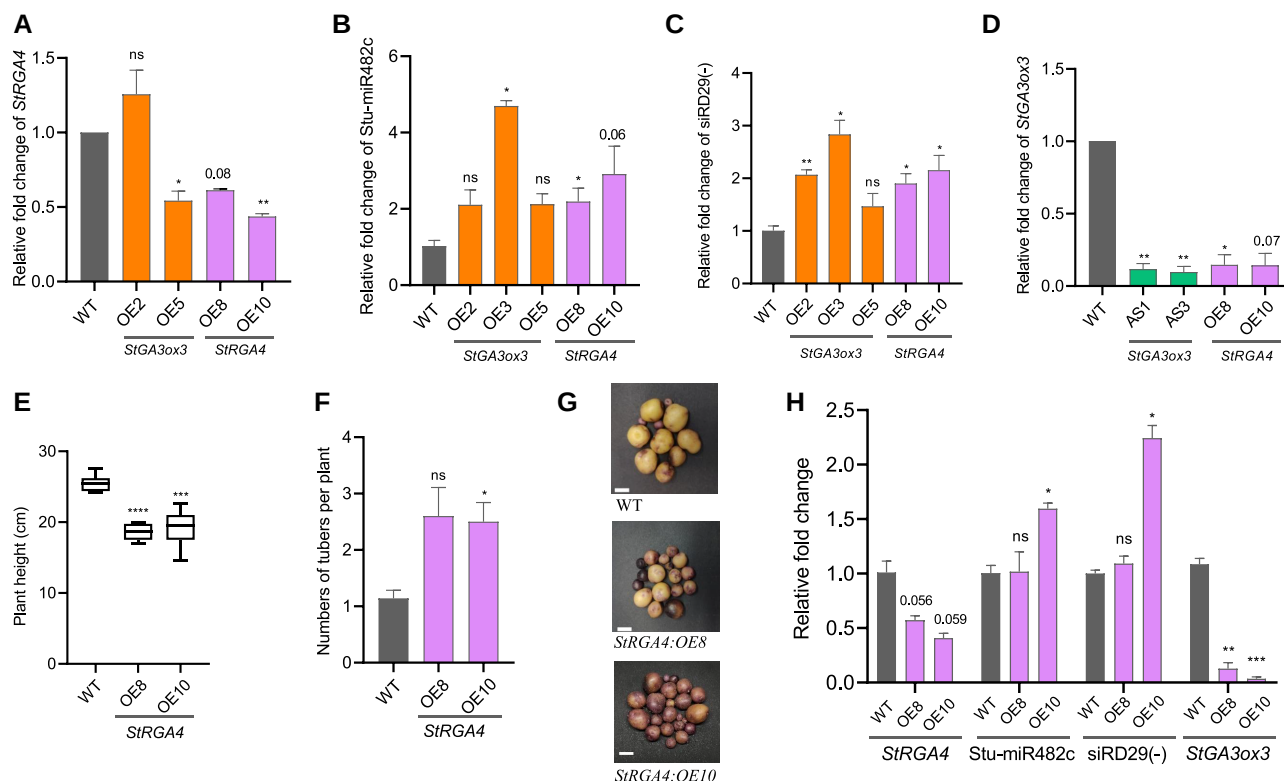


Figure 5. Overexpression lines of *StGA3ox3* and *StGA4* have enhanced levels of *siRD29(-)*, whereas *StGA3ox3*-AS and *StGA4*-OE lines have reduced *StGA3ox3* transcript levels. **A to C**) Expression of the phasiRNA module components, such as *StGA4* (A), *Stu-miR482c* (B), and *siRD29(-)* (C) in leaves of overexpression lines of *StGA3ox3* (OE2, OE3, and OE5) and *StGA4* (OE8 and OE10) compared to WT. **D**) *StGA3ox3* expression in *StGA4::OE* lines and *StGA3ox3::AS* lines (AS1 and AS3) compared to WT. *StEIF3e* was used for normalizing the gene expression data. For **A to D** and **F**, error bars indicate standard error of means (SEM) from 3 biological replicates ($n = 3$). Asterisks (1 and 2) indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively, using Student's *t*-test. ns, not significant at $P > 0.05$. **E**) Plant height for *StGA4*-OE lines compared to WT. Each min to max whisker boxplot is prepared using data from 10 individual plants. Transverse line shows median of datapoints. **F**) Tuber numbers in comparison with WT. Data from 6 independent plants for each line is represented. **G**) Each image contains tubers from 6 plants per line. Image for WT tubers is the same as that in Fig. 4I. Scale bar = 1 cm. **H**) Expression of the module components, *StGA4*, *Stu-miR482c*, *siRD29(-)*, and *StGA3ox3* in the SD-induced (4 days) stolons of *StGA4* (OE8 and OE10) overexpression lines as compared to WT. Error bars indicate standard error of means (SEM) from 3 biological replicates ($n = 3$). For E and H, 1-way ANOVA followed by Dunnett test was performed to check the level of significance. Asterisks (1, 2, and 3) indicate significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. ns, not significant at $P > 0.05$.

of paclobutrazol (GA inhibitor) to the tuber-inducing media (8% sucrose) resulted in the formation of sessile tubers in *StGA3ox3*-OE lines similar to WT (Fig. 6, C and D). To test whether *StGA3ox3* can control tuber stalk development in vivo (in soil), we measured the lengths of the stolons produced by the transgenic lines. *StGA3ox3*-OE lines (#OE2 and #OE3) exhibited higher stolon lengths compared to WT, whereas the stolon lengths in *StGA3ox3*-AS and *StGA4*-OE lines did not vary significantly (Supplemental Fig. S10B). We did not observe a significant increase in the stolon length of the *StGA3ox3* #OE5 line. This may be due to the fact that independent OE lines do not always show similar phenotypes because of the variations in the level of the transgene expression. Our results indicate that *StGA3ox3* controls the tuber stalk development in soil-grown plants, similar to the *in vitro* tuberization assays.

Altered expression of *StGA3ox3* affect GA levels in transgenic lines

To investigate the effect of altered *StGA3ox3* expression on the GA contents, we quantified 5 gibberellins (3 bioactive GAs: GA_3 , GA_4 , and GA_7 ; 1 GA precursor: GA_{12} ; and 1 GA degradation product: GA_8) from the shoot apices of transgenic lines (Table 1). Levels of GA_{12} precursor were increased in *StGA3ox3*-AS and *StGA4*-OE lines. It is known that during GA biosynthesis, GA_{12} can follow either the 13-hydroxylation or non-13-hydroxylation pathway to generate GA_1 , GA_4 , GA_3 , and GA_7 , respectively. The levels of bioactive GA_3 were higher in the *StGA3ox3*-OE line (#OE3). The OE line (#2) though showed an increase in GA_3 content, but it was below the significant threshold. However, *StGA3ox3*-AS lines did not exhibit a change in GA_3 levels. GA_3 levels were significantly less in both of the *StGA4*-OE lines. *StGA3ox3*-OE (#OE3) and *StGA4*-OE (#OE8) lines had higher contents of

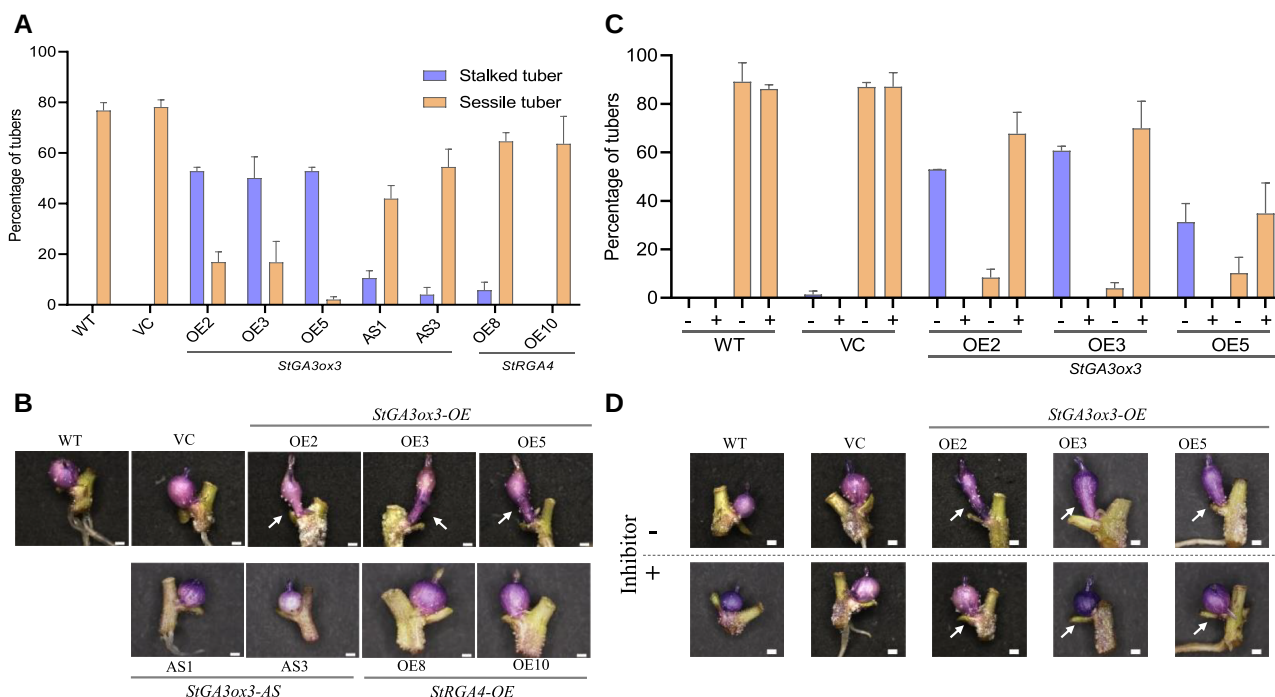


Figure 6. Overexpression and knockdown of *StGA3ox3* influence development of tuber stalk during in vitro tuberization assays. **A)** In vitro tuberization assay. Single-node explants from overexpression lines of *StGA3ox3* (OE2, OE3, and OE5) and *StGA4* (OE8 and OE10) and the antisense lines of *StGA3ox3* (AS1 and AS3) along with wild-type (WT) and vector control (VC) plants were grown on 8% sucrose in dark. The percentage of nodes producing each type of tubers formed in each line was scored on alternate days until the 12th day, and the final tuber number percent is plotted. Error bars indicate standard error of means (SEM) from 3 experimental repeats ($n = 3$), where each experiment was conducted with $n > 80$ nodes per transgenic line. **B)** The nodes with sessile versus stalked tubers were noted during the observations. Mostly, OE lines of *StGA3ox3* produced stalked tubers, whereas antisense lines of *StGA3ox3* or overexpression lines of *StGA4* showed mostly sessile tubers. White arrow indicates the stalk of the tuber. Scale bar = 1 mm. **C)** In vitro tuberization assay using 3 types of transgenic lines with supplementation of a GA biosynthesis inhibitor, paclobutrazol (0.5 mg/L). The symbols (+ and –) indicate the presence or absence of GA inhibitor in the MS media. **D)** Representative images of each type of node with tuber are given. White arrow indicate stalk of the tuber in the presence or absence of GA inhibitor (scale bar = 1 mm).

bioactive GA_4 and GA_7 . We could not determine GA_1 and GA_{20} precursors from the shoot apices. Interestingly, all lines indicated an increase in GA_1 degradation product, GA_8 . Particularly, *StGA4-OE* showed the highest GA_8 content. Gibberellins were also measured from stolons of transgenic lines, but no GA peaks were detected in any of the samples, except for *StGA3ox3-OE* lines, which exhibited low levels of bioactive GAs and higher amount of GA_8 .

StBEL5 (a BEL-like Tf) induces *StGA3ox3* expression in leaves of *promStGA3ox3::GUS* lines

The GUS activity in *promStGA3ox3::GUS* lines was highest in swollen stolons compared to SD-induced stolon. These results suggested that *StGA3ox3* expression in stolons could be regulated through its promoter. The 2.2-kb promoter sequence of *StGA3ox3* was analyzed for the presence of cis-regulatory motifs using tools such as PLANTPAN3.0 and PLACE (Chow et al. 2019; Higo et al. 1999). Several motifs were identified, such as bHLH (59), AP2-ERF (200), bZIP/HD-ZIP (94), MYB (21), MYB/SANT (65) WRKY (253), DOF (356), homeodomain TFs (90), ZF-HD (85), and NAC/NAM (33). Interestingly, 12 tandem TGAC core motifs were also

identified in the *StGA3ox3* promoter using the RSAT tool (Helden et al. 2000) (Fig. 7A). Among them, 6 TGAC core motifs were separated by lesser than 40-bp linkers (Fig. 7A; Supplemental Table S3). To study this regulation, we agroinfiltrated the *StBEL5-OE* gene construct in the leaves of *promStGA3ox3::GUS* lines. As *StBEL5* expression is known to be wound inducible (Chatterjee et al. 2007), we observed an increased expression of *StBEL5* as well as *GUS* in the mock (control) compared to noninfiltrated leaves. RT-qPCR from *StBEL5*-infiltrated leaves showed an increased *GUS* expression (Fig. 7B). GUS protein activity was also increased in *StBEL5-OE* gene construct-infiltrated leaves (Fig. 7C), suggesting that *StBEL5* induces the expression of *StGA3ox3* in potato.

Discussion

Small RNA (sRNA) regulatory networks orchestrate various plant developmental processes, such as initiation of SAM and its maintenance, leaf polarity establishment, flowering time, and root development (Dong et al. 2022). Tuberization in potato is governed by numerous mobile signals, transcription factors, and phytohormones. These factors

Table 1. Levels of GAs (ng/g of freeze-dried tissue weight) in the shoot apices of the transgenic lines (*StGA3ox3-OE*, *StGA3ox3-AS*, and *StGA4-OE*) compared to wild-type (WT) and digena plants after 6 wk in long-day conditions. GAs were quantified using LC-MS/MS with internal standards. The values are means of 3 independent biological replicates ($n = 3$) $\pm$ standard error of means (SEM). One-way ANOVA followed by Dunnett test was performed to check the level of significance. Asterisks (1, 2, 3, and 4) in parenthesis indicate significant differences at $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively

		GA concentrations in shoot apices (ng/g of dried weight) represented as mean $\pm$ SEM				
Plant type		GA3	GA4	GA7	GA8	GA12
Wild type	WT	154.9 $\pm$ 24.2	1132.9 $\pm$ 116.1	16395.7 $\pm$ 1073.3	306.5 $\pm$ 26.2	1052.0 $\pm$ 76.7
<i>StGA3ox3-OE</i>	OE2	384.4 $\pm$ 169.7	1387.8 $\pm$ 294.4	13936.4 $\pm$ 1485.3	385.8 $\pm$ 71.0	1055.2 $\pm$ 107.5
	OE3	561.7 $\pm$ 63.9(*)	1783.0 $\pm$ 186.4	24926.9 $\pm$ 2163.9(*)	496.3 $\pm$ 67.7	1435.7 $\pm$ 67.0
<i>StGA3ox3-AS</i>	AS1	200.7 $\pm$ 26.3	1517.1 $\pm$ 100.1	23570.5 $\pm$ 2170.2	421.6 $\pm$ 23.4	1336.6 $\pm$ 86.8
	AS3	224.3 $\pm$ 30.9	1535.9 $\pm$ 48.4	22428.7 $\pm$ 565.0	422.0 $\pm$ 38.1	1422.7 $\pm$ 72.2
<i>StGA4-OE</i>	OE8	57.2 $\pm$ 3.2(****)	2187.2 $\pm$ 490.0(*)	64234.3 $\pm$ 8146.4(***)	1198.7 $\pm$ 101.5(**)	1968.1 $\pm$ 198.7(***)
	OE10	66.6 $\pm$ 10.3(****)	1090.1 $\pm$ 172.6	42182.6 $\pm$ 3551.5(**)	1105.8 $\pm$ 63.9(**)	1996.6 $\pm$ 104.3(***)

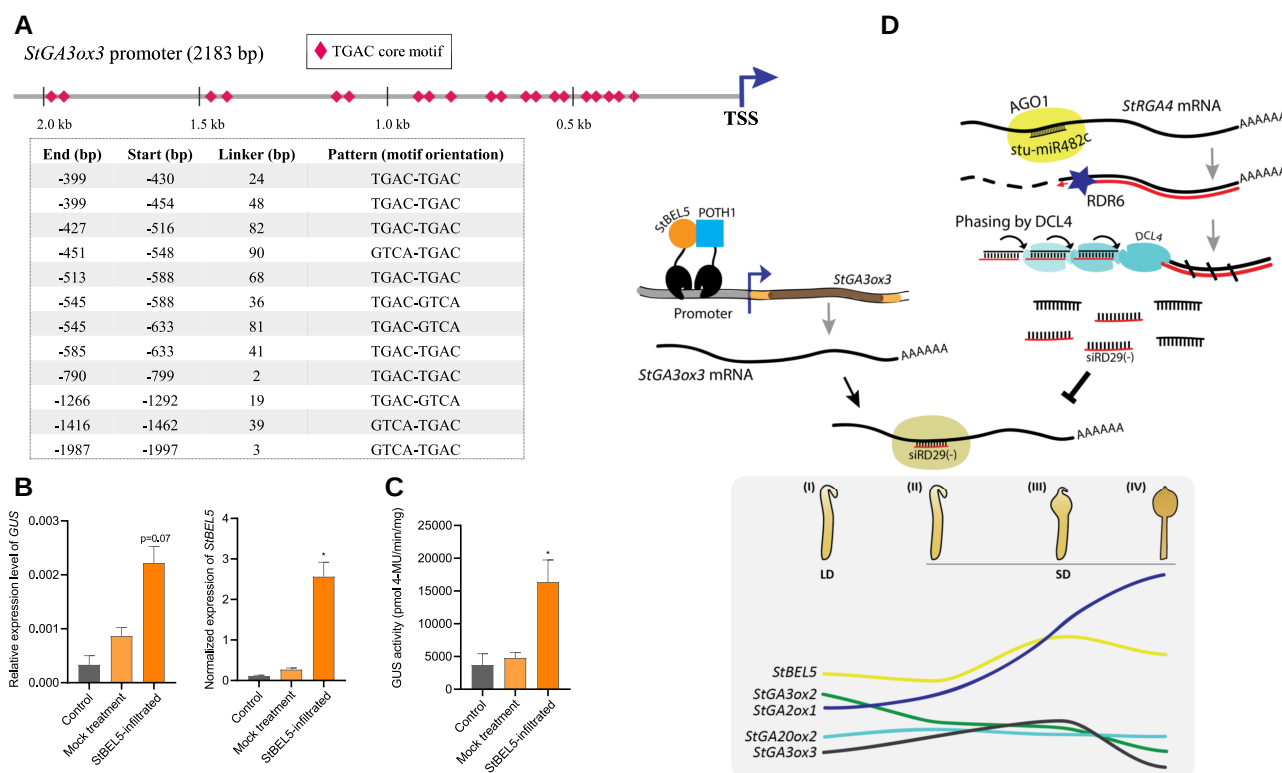


Figure 7. BEL1-like TF *StBEL5* regulates the expression of *StGA3ox3*. **A**) Schematics of *StGA3ox3* promoter highlighting the presence of TGAC core motifs. The promoter sequence (~ 2.1 kb) characterized in Fig. 3 was used to identify the TGAC core motifs (denoted by pink diamond). The detailed location of each motif is given below the schematics and also in Supplemental Table S3. TSS = transcription start site. **B**) Transcript abundance of *GUS* and *StBEL5* in the leaves of *promStGA3ox3::GUS* line agroinfiltrated with 35S::*StBEL5* gene construct. **C**) Quantification of GUS activity in leaves of *promStGA3ox3::GUS* line agroinfiltrated with 35S::*StBEL5* gene construct. Control, uninfiltrated leaves; Mock, buffer infiltrated leaves. Error bars indicate standard error of means (SEM) ($n = 3$). Student's *t*-test was performed to check the level of significance. Asterisks indicate significant difference at $P < 0.05$. **D**) Proposed model for the dual (transcriptional and posttranscriptional) regulation of *StGA3ox3* during stolon-to-tuber transitions of potato. Stolon stages are represented as (I) LD stolon (II) SD stolon, (III) swollen stolon, and (IV) minituber. To highlight the role of *StBEL5* and GA metabolism genes, the expression pattern of *StBEL5*, GA biosynthesis genes (*StGA3ox2*, *StGA3ox3*, and *StGA2ox2*) and the GA catabolism gene *StGA2ox1* are also shown. LD, long day; SD, short day. Arrows indicate activation or promotion and the blunt arrow indicates inhibition.

coordinate a series of developmental events in the stolon to form a mature tuber. However, whether sRNA networks regulate stolon-to-tuber transitions in potato has not been investigated. We have earlier reported that a number of sRNA candidates could potentially be involved in early

stages of tuber development (Kondhare et al. 2018, 2021). Here, we demonstrate the functionality of a gene regulatory module (GRM), *Stu-miR482c-StGA4-siRD29(-)-StGA3ox3*, and its importance during stolon-to-tuber transitions in potato.

Stu-miR482c-StGA4-siRD29(-)-StGA3ox3 GRM is functional in potato

In this study, we provided the experimental evidences for a phasiRNA regulating a GA biosynthesis gene, *StGA3ox3*, in potato. Through transient expression assays in *N. benthamiana*, followed by small RNA sequencing, we showed that Stu-miR482c triggers synthesis of 21-nt phasiRNAs from the *StGA4 PHAS* locus. Further, we demonstrated that 1 of the phasiRNAs from this locus, siRD29(-), is generated at the 29th position from the antisense strand in a RDR6-dependent manner and cleaves *StGA3ox3* transcript in planta (Figs. 1 to 2; Supplemental Fig. S1). These findings establish the fact that Stu-miR482c-StGA4-siRD29(-)-*StGA3ox3* GRM is active. SiRD29(-) is found to be abundant in leaves under LD and was highest in SD-induced stolons (Fig. 1, H and I). Interestingly, siRD29(-) and *StGA3ox3* transcript levels exhibited inverse expression patterns during stolon to swollen stolon stage under SD conditions (Fig. 1I and 3D), indicating the possible role of this module in tuber development.

Earlier, 2 isoforms of GA3ox (referred as *StGA3ox1* and *StGA3ox2*) were reported in potato (Bou-Torrent et al. 2011; Roumeliotis et al. 2013a). However, only *StGA3ox2* has been studied for its role in tuberization (Bou-Torrent et al. 2011; Roumeliotis et al. 2013a). Here, we have identified another isoform of *StGA3ox* (referred as *StGA3ox3*) (Supplemental Fig. S2) and it is a target of siRD29(-) (Fig. 1E). Unique expression patterns of *StGA3ox3* (Fig. 3, A and E) suggest that it could have a distinct role in plant growth and development. Previously, *StGA3ox2* expression was found to be highly reduced in stolons under SD conditions (Bou-Torrent et al. 2011). Surprisingly, we observed the highest expression of *StGA3ox3* in SD-induced swollen stolons (Fig. 3D). It raises an important question: what is the relevance of increased *StGA3ox3* expression during the transitions of a stolon to swollen stolon? A reduced GA level is a prerequisite for tuber development. This perhaps necessitates a regulatory mechanism that could control *StGA3ox3* levels during stolon-to-tuber transitions in potato. For example, in rice, miR9678 has been shown to trigger phasiRNA production from long noncoding RNA WSGAR that could target GA biosynthesis genes to delay seed germination (Guo et al. 2018), suggesting a possible regulation of GA metabolism through phasiRNAs in plants.

Bioactive GAs control leaf expansion and the elongation of stem and root tissues (Davies 2004). We noted that the OE and AS lines of *StGA3ox3* exhibited typical GA-mediated phenotypes (Fig. 4; Supplemental Fig. S6A). Interestingly, *StGA3ox3*-AS lines showed higher tuber numbers compared to WT (Fig. 4H). Previously, a report by Roumeliotis et al. (2013a) showed that *StGA3ox2* RNAi lines had increased tuber numbers (per plant) possibly due to an altered GA:auxin ratio in stolons. However, we did not observe any change in the expression of *StGA3ox2* in *StGA3ox3*-AS lines (Supplemental Fig. S11), suggesting that the phenotypes we

observed were specifically due to the knockdown of *StGA3ox3* only. To identify if pleiotropic phenotypes of *StGA3ox3*-OE lines have any effect on tuber numbers, grafting was conducted. Heterografts (OE/WT, WT/OE) did not show any change in the tuber numbers, suggesting that the leaf morphology of *StGA3ox3*-OE lines may not have any direct effect on the tuberization. However, the unaltered tuber numbers in the heterografts of WT/AS could be the effect of transport of bioactive GAs from leaf to stock (Supplemental Fig. S7). Further, we observed that *StGA4*-OE lines exhibited an increased expression of siRD29(-) in leaves and stolons (Fig. 5, C and H) but reduced the levels of *StGA3ox3* transcripts (Fig. 5, D and H). These lines had quite similar tuber phenotypes as of *StGA3ox3*-AS lines (Fig. 5, F and G) suggesting that the Stu-miR482c-StGA4-siRD29(-) signaling cascade is functional and regulates *StGA3ox3* expression in *StGA4*-OE lines. Hence, it is believable that this GRM is active in leaves as well as in stolons of potato.

Subsequently, we investigated the role of the *StGA3ox3* isoform in the GA biosynthesis pathway. The GA3ox gene family catalyzes the final step in the synthesis of bioactive GAs (GA₁, GA₃, and GA₄) from the GA₂₀ precursor (Hedden and Phillips 2000). Previously, *StGA3ox2* was reported to catalyze GA₁ production (Bou-Torrent et al. 2011). Through transient expression in potato (Supplemental Fig. S9A), we observed an increased GA₁ levels in the potato leaves infiltrated with the *StGA3ox3*-OE construct, whereas GA₃ levels remained unchanged. We noted that the levels of GA₃ and GA₇ were high in *StGA3ox3*-OE lines, whereas *StGA4*-OE lines had significantly reduced GA₃ levels, indicating that *StGA3ox3* might be involved in GA₃ synthesis (Table 1). GA₃ concentration in AS lines of *StGA3ox3* was similar to WT. Also, the expression of *StGA3ox3* was less in the shoot apex (Fig. 3A). These observations led us to speculate that the functions of *StGA3ox3* in the shoot apex of the AS line might be compensated by other GA biosynthesis enzymes. Further in the constitutive *StGA4*-OE lines, *StGA3ox3* transcripts are targeted by siRD29(-); hence, the effect on GA₃ concentration is quite evident in these lines. Even though GA₁ could not be estimated in samples, high levels of GA₈ in *StGA3ox3*-OE lines possibly suggest a metabolic turnover resulted from the breakdown of GA₁ produced. These results, in addition to the *StGA3ox3*-OE phenotypes (such as increased plant height, larger leaf size, reduced chlorophyll, and anthocyanin content), indicate that the *StGA3ox3* isoform could be involved in synthesis of bioactive GA₁ and GA₃.

To dissect the role of *StGA3ox3* in tuber development, we carried out an in vitro tuber induction experiment using *StGA3ox3*-OE, *StGA3ox3*-AS, and *StGA4*-OE lines (Fig. 6). As anticipated, *StGA3ox3*-OE lines exhibited delay in tuberization (Supplemental Fig. S10A); however, noticeable changes were not observed in other transgenic lines. Further, *StGA3ox3*-OE lines produced stalked tubers (Fig. 6, A and B). During in vitro tuberization experiment, application of GA₃ leads to its accumulation in the meristematic region of the nodes that causes cell elongation and formation of

stalked tubers (Shibaoka 1993; Sanz et al. 1996; Xu et al. 1998a). Interestingly, application of paclobutrazol, a GA inhibitor to *StGA3ox3*-OE lines led to the formation of sessile tubers under tuber-inducing in vitro conditions (Fig. 6, C and D), suggesting that enhanced levels of bioactive GA might have stimulated the stalked tuber phenotype. The increased stolon length in *StGA3ox3*-OE lines as compared to WT in soil grown plants (Supplemental Fig. S10B) further supports the notion. A recent study by Kou et al. (2021) reported that GA₃ treatment caused elongation in the flower stock in Chinese cabbage (*Brassica campestris*) via induced expansion of cell walls through activation of cell wall proteins like expansins. These results suggest that *StGA3ox3* could be involved in potato tuber stalk development.

StBEL5 regulates *StGA3ox3* expression in potato

We observed a prominent GUS expression pattern in swollen stolons of *StGA3ox3* promoter lines (Fig. 3O). This directed us to investigate if any transcriptional regulators govern the expression of *StGA3ox3*. BELs interact with KNOTTED1-LIKE (KNOX) TFs to control diverse aspects of plant growth and development (Hay and Tsiantis 2010). The BEL-KNOX heterodimers are shown to regulate the expression of key tuberization genes by binding to the tandem TGAC core motifs in their promoters (Chen et al. 2004; Sharma et al. 2016). The presence of 12 tandem TGAC core motifs on the *StGA3ox3* promoter (Fig. 7A; Supplemental Table S3) indicated a possible regulation by StBEL5. Through transient overexpression of *StBEL5* in the leaves of *promStGA3ox3::GUS* lines, we confirm that StBEL5 acts as an activator of *StGA3ox3* gene expression (Fig. 7, B and C). Increasing *StGA3ox3* levels by StBEL5 is counter intuitive, given the fact that GA inhibits tuberization. The key step in the stolon-to-tuber transition is to regulate the GA:auxin ratio to alter the cell division pattern (Xu et al. 1998b). Increased auxin at the stolon tip is transported to the subapical region of the stolon upon induction (Roumeliotis et al. 2012a, b), resulting in swelling of the stolon. Thus, it appears that StBEL5 induces expression of *StGA3ox3* possibly to maintain the GA:auxin ratio in the tuberizing stolons.

Dual regulation of *StGA3ox3* during stolon-to-tuber transitions

Our findings point toward a possible dual regulation of *StGA3ox3* by 2 independent regulatory pathways—1 is through siRD29(-) and the other is mediated by StBEL5. Obviously, a question arises as to what is the need of such a dual regulation? It is known that at the onset of tuber development, GA levels reduce in the stolon (Xu et al. 1998a). StBEL5, a key tuber marker, inhibits GA biosynthesis gene *StGA20ox1* (Chen et al. 2004) but upregulates GA catabolism gene *StGA2ox1* (Sharma et al. 2016) to reduce GAs in the stolon. In the present investigation, we found that siRD29(-) levels are high in SD-induced stolons of wild-type andigena plants (Fig. 11). Further, *StRGA4*-OE lines exhibited

a 2-fold increase in siRD29(-) levels and showed phenotypes similar to *StGA3ox3*-AS lines, suggesting that enhanced siRD29(-) levels may exert a control to reduce *StGA3ox3* abundance in the SD-induced stolon (Fig. 3D). However, we found induced expression of *StGA3ox3* in swollen stolons, implicating that the control exerted by siRD29(-) to reduce *StGA3ox3* levels is possibly overridden by other gene regulatory mechanisms in the swollen stolon. We noticed that a tuberization activator *StBEL5* and *StGA3ox3* are abundantly expressed in the swollen stolon (Fig. 7; Supplemental Fig. S3, B and E), suggesting a possible regulation of *StGA3ox3* by StBEL5. Indeed, we showed that StBEL5 increases *StGA3ox3* expression in potato (Fig. 7B). As optimal GA levels are required for stolon-to-tuber transitions, it emphasizes the need for a distinct mechanism for spatiotemporal control of GA levels in the stolon, which is possibly achieved either independently by siRD29(-) and StBEL5 or in combination. The identification of such gene regulatory module to regulate an important phytohormone GA suggests that it is crucial for a plant to make developmental decisions to achieve the ultimate outcome. Our current study proposes a GRM that could regulate potato tuber development. What signals activate this module is an interesting question. Moreover, whether bioactive GAs could exhibit any feedback mechanism to maintain the phytohormone ratios in stolons is not yet investigated. Notably, we show that this GRM is conserved in tomato (*Solanum lycopersicum*) (Supplemental Fig. S12), indicating the relevance of such modules in developmental processes.

In conclusion, we show that a regulatory network of *Stu-miR482c-StRGA4-SiRD29(-)-StGA3ox3* is functional and maintains *StGA3ox3* levels during stolon to swollen stolon transition. This module provides flexibility to the regulation of bioactive GA levels in fine tuning the stolon-to-tuber development process in potato.

Materials and methods

Plant materials and growth conditions

In this study, the photoperiod-responsive potato cultivar—*S. tuberosum* ssp. *andigena* 7540—which tuberizes under short-day conditions (SD; 8 h light/16 h dark) was used. WT andigena plants were propagated by subculturing stem nodal explants on Murashige and Skoog's (MS) media supplemented with 2% sucrose (w/v) (Murashige and Skoog 1962). In vitro potato plants were maintained in a plant growth incubator (Percival Scientific) at 24°C under long-day (LD; 16 h light/8 h dark) conditions. *N. benthamiana* plants (age: 5 to 6 wk after seed germination in soil) were used for *Agrobacterium tumefaciens*-mediated transient coinfiltration assays.

Gene expression analysis

For studying transcript accumulation, 4 tissue types (shoot apex, leaf, node, and stem) were harvested from 2-wk-old soil-grown andigena plants. To investigate the effect of the

SD photoperiod, plants were grown in soil under LD conditions for 10 wk until they attained 10 to 12 leaves. Subsequently, half of the plants were transferred to the tuber-inducing SD conditions, whereas the remaining plants were maintained under LD photoperiod. Tissue types (shoot apex, leaf, node, and stem) along with samples comprising stolon-to-tuber transition stages (stolon, swollen stolon, and minituber) were harvested at 10 and 14 days after their transfer to SD. Leaves were also harvested on the earlier time-points (4th and 7th days after SD induction). All samples were collected between Zeitgeber time (ZT) = 2 and ZT = 4. Total RNA was isolated from 100 mg of crushed tissues using RNAiso Plus (DSS TaKaRa) as per the manufacturer's instructions. Complementary DNA (cDNA) synthesis was carried out using 2 μ g of total RNA, Superscript IV Reverse Transcriptase (SSIV-RT Invitrogen), and Oligo(dT) primers. For gene expression analysis, reverse transcription quantitative PCR (RT-qPCR) was performed on a CFX96 Real-Time System (Bio-Rad) using gene-specific primers (Supplemental Table S4). Reactions were prepared with SYBR green master mix (TaKaRa Clontech) and incubated at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, gene-specific annealing temperature for 15 s, and extension at 72°C for 15 s. Similarly, 2 μ g of total RNA was reverse transcribed using stem loop primers, to study sRNA expression as per the protocol by Varkonyi-Gasic et al. (2007). RT-qPCR was performed with sRNA-specific forward and universal reverse primers (Supplemental Table S4). PCR specificity was checked by melting curve analysis and data were analyzed using the $2^{-\Delta C_t}$ or $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Cleavage site mapping by 5' RLM-RACE assay

To validate targets of small RNAs, a modified 5' RNA Ligase-Mediated Rapid Amplification of cDNA Ends (5' RLM-RACE) assay was performed using the First Choice RLM-RACE kit (Ambion). Briefly, total RNA was extracted from stolons of WT potato (*andigena*) plants by TRIzol reagent and ligated to the 5' RNA adapter. cDNA synthesis was performed using gene-specific reverse primers (Supplemental Table S4). Two sets of PCRs were performed with adapter-specific forward primers and gene-specific reverse primers (Supplemental Table S4). PCR amplicons from secondary-nested PCRs were cloned in a subcloning vector pGEM-T-easy and sequence verified to identify the cleavage sites of sRNAs on their target gene transcripts.

Phylogenetic analysis

The amino acid sequences of putative StGA3ox isoforms were retrieved from Arabidopsis (*A. thaliana*), potato, tobacco (*Nicotiana tabacum*), and tomato (*S. lycopersicum*) genomes. The phylogenetic tree was constructed using T-COFFEE (hRp://www.ch.embnet.org/soaware/TCoffee.html) and represented with TreeDyn (v198.3) (Dereeper et al. 2008).

Gene construct designs and generation of potato transgenic lines

To generate constitutive overexpression (OE) gene constructs, the full-length coding sequences of StGA3ox3 (1313 bp) and StGA4 (3239 bp) were amplified by reverse transcription PCR from the leaves of soil-grown *andigena* plants using primers listed in Supplemental Table S4. These sequences were then individually mobilized into the binary vectors, such as pBI121 and pCambia1300, downstream to a constitutive CaMV35S promoter. Henceforth, these constructs are referred as 35S::StGA3ox3-pBI121 and 35S::StGA4-pCambia1300. Similarly, the Stu-miR482c precursor OE construct was generated in a binary vector pBI121 (referred as 35S::pre-miR482c-pBI121). For the antisense construct, a nonconserved 446-bp sequence of StGA3ox3 (comprising 41 bp from 5' untranslated region [UTR] region and 401 bp upstream to 5' UTR) was cloned in an antisense direction downstream to the CaMV35S promoter into a binary vector pBI121. To analyze StGA3ox3 promoter activity, ~2.3 kb sequence upstream of the StGA3ox3 gene (including 5' UTR of 41 bp) was amplified from genomic DNA of *andigena* plants and cloned upstream to the GUS reporter gene (*uidA*) in the pBI101 to generate the *promStGA3ox3::GUS*-pBI101 construct. All the gene constructs were then mobilized into *A. tumefaciens* strain GV2260. Transgenic potato plants were generated using the *Agrobacterium*-mediated leaf transformation method (Banerjee et al. 2006). The transgenic line of *andigena* harboring 35S::GUS construct was considered as a vector control (VC). Phenotypic characteristics, such as plant height, internodal distance, leaf length, and shape in transgenic lines, were recorded before the initiation of the SD photoperiod, whereas the tuber and root biomass were measured after 4 wk of SD induction. Moreover, the StBEL5-coding sequence (2,064 bp) was PCR amplified from cDNA of *S. tuberosum* ssp. *andigena* (Supplemental Table S4) and into the pBI221 vector to generate the StBEL5 OE construct.

Agrobacterium-mediated coinfiltration assays

To understand in planta phasiRNA production, *Agrobacterium*-mediated coinfiltration assays were performed in leaves of 6-wk-old WT and RDR6 mutant plants of *N. benthamiana*. 35S::StGA4-pCambia1300 and 35S::pre-miR482c-pBI121 constructs were transformed into the *A. tumefaciens* GV3101 strain. For coinfiltration assays, individual *Agrobacterium* colony harboring respective gene construct was inoculated in 3-mL LB broth containing appropriate antibiotics and grown overnight (primary culture). One hundred-milliliter secondary cultures (having 150 μ M acetosyringone) were set up without any antibiotics. When the secondary culture reached an OD₆₀₀ of 1.00, cells were centrifuged and pellet was resuspended in a buffer (10 mM MgCl₂, 10 mM MES-K; pH = 5.6, plus 150 μ M acetosyringone), incubated at room temperature on a tube roller for 3 hrs, followed by coinfiltration into the leaves of *N. benthamiana* plants using a needleless syringe.

Coinfiltrations with *Agrobacteria* containing pBI121 and pCAMBIA1300 served as vector controls. Leaves were harvested following 48 hrs of coinfiltrations and subjected further for sRNA analysis. For in planta validation of StGA3ox3 as a target of siRD29(-), coinfiltrations were carried out in the leaves of WT *N. benthamiana* plants with 35S::StGA4-pCAMBIA1300, 35S::pre-miR482c-pBI121, and 35S::StGA3ox3-pBI121 constructs.

To determine whether StBEL5 regulates StGA3ox3 gene expression, *Agrobacterium*-mediated coinfiltration assays were performed in leaves of *promStGA3ox3::GUS* lines (3- to 4-wk-old soil-grown plants). The 4th leaf of each plant was infiltrated with the 35S::StBEL5 gene construct. Leaves were harvested 48 hrs post-infiltration and processed for GUS transcript accumulation and protein activity.

Dot blot analysis

Dot blot was carried out for sRNAs. Briefly, RNAs (2.5, 5, and 10 μ g) were resuspended in deionized formamide (Merck) heated at 98°C for 2 min. The sample (2 μ L) was loaded on the hybond N⁺ membrane (Ameshram) several times till the requisite amount of RNA was obtained on a single dot area. Further, the blot was UV crosslinked and hybridization was performed at 35°C for 24 hrs in UltraHyb-Oligo buffer (Ambion) with the oligo probes (Supplemental Table S4) end labeled with ³²P by poly-nucleotide kinase (Invitrogen). Blots were washed with 2× SSC buffer + 0.1% SDS (w/v) thrice for 15 min at 35°C. Blots were exposed to a phosphor screen for 5 days, and the signal was captured using Typhoon phosphor imager (GE healthcare).

Analysis of StGA3ox3 promoter activity

To analyze StGA3ox3 promoter activity, 4 *promStGA3ox3-GUS* transgenic andigena lines were generated. All the lines showed a similar GUS activity pattern across the tissue types. Of these, 1 representative line was chosen for further analysis. Plants for the *promStGA3ox3-GUS* transgenic line were grown in vitro for 5 wk under LD conditions before transferring to the soil. They were grown under LD conditions until they attained 10 to 12 leaves. Half of the plants were subjected to SD induction for 14 days, while the remaining half continued to grow under LD. Stolon-to-tuber transition stages as well as in vitro grown plantlets (4 wk old) were used to perform GUS assay as described in Jefferson (1987). Briefly, samples were kept for overnight in GUS extraction buffer at 37°C and transferred to the series of ethanol gradients from 20% to 100% (v/v) for the complete removal of chlorophyll. Samples were photographed in a stereo microscope (Olympus MVX10). Fluorometric MUG analysis from the subset of tissue samples (mentioned above) was performed as described (Jefferson et al. 1987). Frozen samples were homogenized in GUS extraction buffer (100 μ L). Samples were centrifuged at 16,000 g for 5 min on a benchtop centrifuge. Clear supernatant was removed and used for protein estimation by Bradford's method (Bradford 1976). Aliquots of extracts with approximately 5 μ g of protein were made, and

their volume was made up to 150 μ L with extraction buffer. GUS assay buffer (5 μ L) was added to each sample and incubated at 37°C for 3 h. Reaction was stopped by adding stop (0.2 M Na₂CO₃) buffer. Fluorescence of the samples was measured using a multimode plate reader (Ensign, PerkinElmer) with excitation wavelength 365 nm and emission wavelength 455 nm.

Histology and microscopy

To visualize the cell type-specific GUS expression, the tissues were fixed in 0.1 M sodium phosphate buffer containing 4% paraformaldehyde (w/v) (Sigma) and 2% glutaraldehyde (v/v) overnight at 4°C (Barker et al. 2021). The fixed tissues were then dehydrated in a series of ethanol gradient from 20% to 100% (v/v), followed by washes with xylene (JT Baker). Tissue blocks were prepared with molten Paraplast (Leica). Ten-micrometer sections were cut with the help of Microtome (Leica) and mounted with DPX on glass slides. Peels from the abaxial side of GUS-stained leaves were mounted with 10% (v/v) glycerin solution to visualize guard cells.

Estimation of chlorophyll and anthocyanin content

Terminal leaflet of the 5th leaf (from the shoot apex) was excised from plants 4 wk after soil transfer for the purpose of chlorophyll and anthocyanin estimation. Using 100 mg of freeze-dried leaf tissues, chlorophyll was extracted in 80% acetone (v/v) and the absorbance of supernatant was measured at 645 and 663 nm wavelengths, and the total chlorophyll content (mg g⁻¹) was calculated per the report of Bhattacharjee and Sharma (2012). Anthocyanin estimation was performed with a protocol described by Nakata and Ohme-Takagi (2014). Five volumes of extraction buffer (45% methanol [v/v] and 5% acetic acid [v/v]) were added to the 100 mg of plant tissues and mixed thoroughly. Samples were centrifuged at 12,000 g for 10 min at room temperature and the absorbance (Abs) of the supernatant was measured at 530 and 657 nm and the anthocyanin content, expressed as Abs 530 g FW⁻¹.

In vitro tuberization

A modified version of the method described by Hendriks et al. (1991) was used for in vitro tuberization assays. Single-node cuttings of WT andigena, VC, and 3 types of transgenic potato lines (StGA3ox3-OE, StGA3ox3-AS, and StGA4-OE) were grown in vitro for 4 wk under LD conditions on MS media containing 2% sucrose (w/v) and 0.2% phytagel (w/v) (Sigma-Aldrich). Four consecutive nodes from the apex (excluding apical region) containing a dormant single axillary bud from 4-wk-old in vitro grown plants were transferred to the tuber-inducing MS media with 8% sucrose (w/v) and 0.2% phytagel (w/v) and incubated in the dark at 18°C for 2 wk, and a number of tubers formed were scored on alternate days. For each independent line, more than 80 single nodes ($n > 80$) were used. Moreover, to study the effect of GA inhibitor treatment, 48 single nodes

of transgenic lines, WT, and VC plants were incubated on tuber-inducing medium supplemented with GA biosynthesis inhibitor—paclobutrazol (0.5 mg/L) or mock (control)—and scored for the rate of tuber formation.

Small RNA sequencing

To identify the phase of siRD29(-) generation, 5th leaves of *N. benthamiana* WT plants were coinfiltrated with *Agrobacterium* containing either 35S::StRGA4-pCambia1300 and 35S::pre-miR482c-pBI121 or only 35S::StRGA4-pCambia1300. The leaves coinfiltrated with pBI121 and pCambia1300 served as vector controls. Total RNA was isolated from these samples as described before. For small RNA sequencing, all 3 coinfiltrated samples (35S::StRGA4-pCambia1300 + 35S::pre-miR482c-pBI121, 35S::StRGA4-pCambia1300, and pBI121 + pCambia1300) were processed in duplicates. RNA integrity was checked on Agilent Bioanalyzer Chip (Tapestation 2200). Six independent small RNA libraries were prepared and subjected to the deep sequencing.

Processing of sRNA reads for identification of phasiRNAs

Sequencing reads of 51 bp in length were generated with the Illumina HiSeq-X sequencing platform (Bionivid Technology Pvt. Ltd.). The quality of raw reads was examined by NGSQCToolkit. Raw reads were filtered for high quality with a Q-score ≥ 20 . rRNA and tRNA reads were filtered out by the sRNA workbench, and adaptor null reads were discarded. Redundant reads from coinfiltrated samples were used to identify the PHAS locus with the help of sRNAworkbench (version 4.7), keeping a cut-off of at least 2 abundances for each aligned read with a length preference to 21 and a P-value cut-off of 0.05.

Quantification of GAs from agroinfiltrated potato leaves

For the quantification of GA levels (GA₁, GA₃, and GA₄), a modified version of the protocol (Shaikh et al. 2022) was followed. To test if StGA3ox3 is involved in the production of GA₁, GA₃, or GA₄, *Agrobacterium*-mediated coinfiltration assays were performed in leaves of 4-wk-old soil-grown WT plants. The 4th leaf was agroinfiltrated with the 35S::StGA3ox3 construct, and samples were collected 48 hrs after infiltration. Liquid N₂-pulverized tissue (100 mg) was extracted in 250 μ L of 80% methanol (v/v) containing 400 ng/mL of Adonitol as an internal standard to monitor response of the machine throughout the run. Samples were vortexed vigorously and centrifuged twice at 14,000 rpm for 30 min at 4°C. Extracts were ran using the Phenomenex Gemini C18 Column (50 $\times$ 4.6 mm, 5 μ m, 110 Å) in an AB Sciex X500R Ultra-Performance Liquid Chromatography-Electrospray Ionization-Quadrupole Time of Flight (UPLC-ESI-QTOF) Mass Spectrometer, in a negative ionization mode with a flow rate of 0.5 mL min⁻¹ and a gradient solvent program of 16 min (0.0 min, 10% methanol/water; 3.0 min, 10% methanol/water; 6.0 min, 50% methanol/water; 7.0 min, 70% methanol/water; 10.0 to 12.0 min, 95% methanol/water;

and 13.0 to 16.0 min, 5% methanol/water). Formic acid (0.1% [v/v]; LC-MS grade) was added to both the solvents. For MS/MS, the following parameters, ion spray voltage (V): -4500, declustering potential (V): -50 V, and collision energy: -35 V were used.

Gibberellin quantification from transgenic lines

Shoot apices as well as stolons of transgenic lines (StGA3ox3-OE, StGA3ox3-AS, and StRGA4-OE) and WT plants grown in soil for 6 wk under long-day conditions were used for GA estimation. GAs were extracted according to published protocol (Simura et al. 2018; Jathar et al. 2022; Kundu et al. 2022) at NIPGR Metabolomics Facility, New Delhi. The lyophilized plant sample (35 mg) was extracted with 1 mL of cold extraction buffer (MeOH: H₂O: HCOOH, 15:4:0.1) consisting with 20 ng of [²H₂] GA₄ as an internal standard. The homogenized sample was centrifuged at 12,000 g for 10 min at 4°C. Supernatant was taken out and loaded onto a Strata-X (Phenomenex) C₁₈ solid phase extraction (SPE) column preconditioned with 1 mL of methanol and 1 mL of 0.1% formic acid (v/v) in water. After loading, the SPE column was washed twice with 0.1% formic acid (v/v) and 5% methanol. The sample was eluted with 1 mL 0.1% (v/v) formic acid in acetonitrile and dried in SpeedVac. Dried sample was resuspended in 50 μ L 5% (v/v) methanol and analyzed by liquid chromatography coupled with a SCIEX 6500⁺ triple-quadrupole-trap MS/MS. LC-MS/MS was performed. Separation of GAs (GA₁, GA₃, GA₄, GA₇, GA₈, GA₁₂, and GA₂₀) was carried out with a Zorbax Eclipse XDB C₁₈ column (50 $\times$ 4.6 mm, 1.8 μ m, Agilent Technologies). The mobile phase comprised solvent A (water, 0.05% formic acid) and solvent B (acetonitrile) with the following elution profile: 0 to 0.5 min, 5% B; 0.5 to 5 min, 95% B; 5.5 to 5.6 min 95% to 5% B; and 5.6 to 7.5 min 5% B, with a flow rate of 0.5 mL min⁻¹. The column temperature was maintained at 27°C. For detection, the mass spectrometer was operated in negative ionization mode (MRM modus) to monitor analyte parent ion→product ion transition (345→143 for GA₃, 331→213 for GA₄, 329→211 for GA₇, 363→257 for GA₈, 331→313 for GA₁₂, 331.1→243 for GA₂₀, and 333.4→259.2 for [²H₂] GA₄). Mass spectrometer settings were as follows: ion spray voltage, -4,500 eV; turbo gas temperature, 500°C; nebulizing gas, 50 psi; curtain gas, 45 psi; and target scan time of 280 msec. Analyst 1.7 software (Applied Biosystems) was used for data acquisition and processing.

Grafting

StGA3ox3-OE (#OE3), StGA3ox3-AS (#AS1) lines, and WT plants were maintained in vitro on MS medium for 3 wk before transferring to the soil. Lines were grown in soil for 4 wk (16 h light, 22°C/8 h dark, 20°C). Thereafter, 3 combinations of homografts (WT/WT; OE/OE and AS/AS) and 4 types of heterografts (WT/OE; OE/WT; WT/AS and AS/WT) were prepared as per the protocol described earlier (Bhögale et al. 2014). Grafts were incubated for 15 days in LD for

recovery before being transferred to SD conditions for 4 wk and scored for the tuberization phenotype.

Statistical analysis

Student's *t*-test or 1-way ANOVA analysis were performed for the experiments. To determine individual statistical differences, Dunnett or Tukey HSD post hoc tests were carried out, following 1-way ANOVA. One, 2, 3, and 4 asterisks indicate $P < 0.05$, <0.01 , <0.001 , and <0.0001 , respectively. Error bars represent standard error of means (SEM). Min to max boxplots were plotted in a way that the whiskers of the boxplot included the smallest value and up to the largest value. All data points were included for statistical analysis.

Accession numbers

Sequence data from this article can be found in the PGSC database (<http://spudb.uga.edu>) under accession numbers: *StGA4* (Soltu.DM.09G019040.1), *StGA3ox3* (Soltu.DM.01G018850.1), *StBEL5* (Soltu.DM.06G029500.1), *StGA2ox1* (Soltu.DM.02G013470.1), *StGA2ox2* (Soltu.DM.06G012790.1), and *StGA3ox2* (Soltu.DM.02G013430.1).

Acknowledgments

We are thankful to Sir. Prof. David Baulcombe (University of Cambridge) for providing us the seeds of *RDR6-RNAi* lines of *N. benthamiana* (obtained with kind permission from Dr. P. V. Shivaprasad, NCBS, Bangalore, India). Phytohormone GA standards are a kind gift from Prof. Peter Hedden (Emeritus Professor, Rothamsted Research, UK). We sincerely thank NIPGR Metabolomics Facility, Delhi, India, and Dr. Jyothilakshmi Vadassery for their help in GA analysis. Authors appreciate help from Ms. Nikita Patil in designing the model illustrated in Fig. 7 and critical reading of the manuscript. Authors thank Mr. Nitish Lahigude for his help in plant maintenance in the growth chambers and greenhouse.

Author contributions

N.N.M., K.R.K., and A.K.B. planned the experiments and wrote the manuscript. N.N.M. constructed all the vectors, generated transgenic lines, and carried out phenotypic and transcriptomic data analyses. K.R.K. helped in conducting experiments and troubleshooting, interpreted the data, and edited the manuscript. K.S. characterized the promoter lines and generated microscopy data. M.M. and K.S. conducted dot blot experiments. A.K.B. conceived the project under study, obtained funding and necessary resources, interpreted data sets, and supervised the entire study. All authors have read and approved the final manuscript.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Table S1. Small RNA sequencing of stolon-to-tuber transition stages from andigena and identification of *TAS-like* loci *StGA4*.

Supplemental Table S2. List of siRNAs generated from *StGA4 PHAS* locus post *Stu-miR482c* cleavage.

Supplemental Table S3. List of 12 tandem TGAC core motifs present within 2.2 kb upstream sequence from TSS of *StGA3ox3* gene.

Supplemental Table S4. List of primers used in this study.

Supplemental Figure S1. Detection of siRD29(-) from SD-induced stolon and its confirmation by Sanger sequencing.

Supplemental Figure S2. Phylogenetic relationship of GA3ox proteins.

Supplemental Figure S3. Expression analysis of GA metabolism genes during stolon-to-tuber transitions under SD.

Supplemental Figure S4. Quantification of GUS activity from *promStGA3ox3::GUS* lines during stolon-to-tuber transitions under short-day (SD) conditions.

Supplemental Figure S5. Relative fold difference in the expression of *StGA3ox3* in OE and AS lines.

Supplemental Figure S6. Phenotypic traits of *StGA3ox3* OE, AS, and *StGA4* OE lines.

Supplemental Figure S7. Tuber phenotype in grafts generated from *StGA3ox3* overexpression (OE), antisense (AS) lines, and WT.

Supplemental Figure S8. Expression analysis for the module components in SD-induced stolons of *StGA3ox3* overexpression (OE) and antisense (AS) lines.

Supplemental Figure S9. Quantification of gibberellin (GA_1) levels from leaves of potato plant infiltrated with *Agrobacterium* containing 35S::*StGA3ox3* gene construct.

Supplemental Figure S10. Effect of *StGA3ox3* expression on tuber formation during in vitro tuberization experiment and stolon length in soil-grown plants.

Supplemental Figure S11. Expression of *StGA3ox2* transcripts in *StGA3ox3* overexpression (OE), antisense (AS), and *StGA4* OE lines.

Supplemental Figure S12. Conservation of *Stu-miR482c-StGA4-siRD29(-)-StGA3ox3* gene regulatory module in tomato.

Funding

This work was supported by the Science and Engineering Research Board (SERB), India grant (# CRG/2021/002283), and the core funding from the Indian Institute of Science Education and Research Pune (IISER Pune). N.N.M. sincerely acknowledges the research fellowship from the Council of Scientific and Industrial Research, India.

Conflict of interest statement. None declared.

Data availability

The raw sequencing data were deposited in the NCBI Short Read Archive (SRA) database (<http://www.ncbi.nlm.nih.gov/sra/>). It can be retrieved using the accession number PRJNA931820.

References

- Allen E, Xie Z, Gustafson AM, Carrington JC. MicroRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell*. 2005;**121**(2):207–221. <https://doi.org/10.1016/j.cell.2005.04.004>
- Banerjee AK, Prat S, Hannapel DJ. Efficient production of transgenic potato (*S. tuberosum* L. ssp. *andigena*) plants via *Agrobacterium tumefaciens*-mediated transformation. *Plant Sci J*. 2006;**170**(4):732–738. <https://doi.org/10.1016/j.plantsci.2005.11.007>
- Barker R, Fernandez Garcia MN, Powers SJ, Vaughan S, Bennett MJ, Phillips AL, Thomas SG, Hedden P. Mapping sites of gibberellin biosynthesis in the Arabidopsis root tip. *New Phytol*. 2021;**229**(3):1521–1534. <https://doi.org/10.1111/nph.16967>
- Bhattacharjee S, Sharma GD. Effect of dual inoculation of arbuscular mycorrhiza and rhizobium on the chlorophyll, nitrogen and phosphorus contents of pigeon pea (*Cajanus cajan* L.). *Adv Microb*. 2012;**2**(4):25945. <https://doi.org/10.4236/aim.2012.24072>
- Bhogale S, Mahajan AS, Natarajan B, Rajabhoj M, Thulasiram HV, Banerjee AK. MicroRNA156: a potential graft-transmissible microRNA that modulates plant architecture and tuberization in *Solanum tuberosum* ssp. *andigena*. *Plant Physiol*. 2014;**164**(2):1011–1027. <https://doi.org/10.1104/pp.113.230714>
- Bonar N, Liney M, Zhang R, Austin C, Dessoly J, Davidson D, Stephens J, McDougall G, Taylor M, Bryan GJ, et al. Potato miR828 is associated with purple tuber skin and flesh color. *Front Plant Sci*. 2018;**9**:1742. <https://doi.org/10.3389/fpls.2018.01742>
- Borges F, Martienssen RA. The expanding world of small RNAs in plants. *Nat Rev Mol Cell Bio*. 2015;**16**(12):727–741. <https://doi.org/10.1038/nrm4085>
- Bou-Torrent J, Martínez-García JF, García-Martínez JL, Prat S. Gibberellin A1 metabolism contributes to the control of photoperiod-mediated tuberization in potato. *PLoS One*. 2011;**6**(9):e24458. <https://doi.org/10.1371/journal.pone.0024458>
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;**72**(1–2):248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
- Carrera E, Bou J, García-Martínez JL, Prat S. Changes in GA20-oxidase gene expression strongly affect stem length, tuber induction and tuber yield of potato plants. *Plant J*. 2000;**22**(3):247–256. <https://doi.org/10.1046/j.1365-313x.2000.00736.x>
- Chatterjee M, Banerjee AK, Hannapel DJ. A BELL1-like gene of potato is light activated and wound inducible. *Plant Physiol*. 2007;**145**(4):1435–1443. <https://doi.org/10.1104/pp.107.105924>
- Chen H, Banerjee AK, Hannapel DJ. The tandem complex of BEL and KNOX partners is required for transcriptional repression of ga20ox1. *Plant J*. 2004;**38**(2):276–284. <https://doi.org/10.1111/j.1365-313X.2004.02048.x>
- Chen HM, Chen LT, Patel K, Li YH, Baulcombe DC, Wu SH. 22-Nucleotide RNAs trigger secondary siRNA biogenesis in plants. *Proc Natl Acad Sci U S A*. 2010;**107**(34):15269–15274. <https://doi.org/10.1073/pnas.1001738107>
- Chow CN, Lee TY, Hung YC, Li GZ, Tseng KC, Liu YH, Kuo PL, Zheng HQ, Chang WC. PlantPAN3.0: a new and updated resource for reconstructing transcriptional regulatory networks from ChIP-seq experiments in plants. *Nucleic Acids Res*. 2019;**47**(D1):D1155–D1163. <http://dx.doi.org/10.1093/nar/gky1081>
- Davies PJ. Plant hormones: biosynthesis, signal transduction, action. Dordrecht Zuidholland, Netherlands: Springer Science & Business Media; 2004.
- Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Guindon S, Lefort V, Lescot M, Claverie JM, et al. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res*. 2008;**36**(Web Server):W465–W469. <https://doi.org/10.1093/nar/gkn180>
- Dong Q, Hu B, Zhang C. microRNAs and their roles in plant development. *Front Plant Sci*. 2022;**13**:824240. <https://doi.org/10.3389/fpls.2022.824240>
- Fei Q, Xia R, Meyers BC. Phased, secondary, small interfering RNAs in posttranscriptional regulatory networks. *Plant Cell*. 2013;**25**(7):2400–2415. <https://doi.org/10.1105/tpc.113.114652>
- Guo G, Liu X, Sun F, Cao J, Huo N, Wuda B, Xin M, Hu Z, Du J, Xia R, et al. Wheat miR9678 affects seed germination by generating phased siRNAs and modulating abscisic acid/gibberellin signaling. *Plant Cell*. 2018;**30**(4):796–814. <https://doi.org/10.1105/tpc.17.00842>
- Hannapel DJ, Sharma P, Lin T, Banerjee AK. The multiple signals that control tuber formation. *Plant Physiol*. 2017;**174**(2):845–856. <https://doi.org/10.1104/pp.17.00272>
- Hay A, Tsiantis M. KNOX genes: versatile regulators of plant development and diversity. *Development*. 2010;**137**(19):3153–3165. <https://doi.org/10.1242/dev.030049>
- Hedden P, Phillips AL. Gibberellin metabolism: new insights revealed by the genes. *Trends Plant Sci*. 2000;**5**(12):523–530. [https://doi.org/10.1016/S1360-1385\(00\)01790-8](https://doi.org/10.1016/S1360-1385(00)01790-8)
- Helden JV, Olmo MLD, Pérez-Ortín JE. Statistical analysis of yeast genomic downstream sequences reveals putative polyadenylation signals. *Nucleic Acids Res*. 2000;**28**(4):1000–1010. <https://doi.org/10.1093/nar/28.4.1000>
- Hendriks T, Vreugdenhil D, Stiekema WJ. Patatin and four serine proteinase inhibitor genes are differentially expressed during potato tuber development. *Plant Mol Biol*. 1991;**17**(3):385–394. <https://doi.org/10.1007/BF00040633>
- Higo K, Ugawa Y, Iwamoto M, Korenaga T. Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res*. 1999;**27**(1):297–300. <http://dx.doi.org/10.1093/nar/27.1.297>
- Jathar V, Saini K, Chauhan A, Rani R, Ichihashi Y, Ranjan A. Spatial control of cell division by GA-OsGRF7/8 module in a leaf explaining the leaf length variation between cultivated and wild rice. *New Phytol*. 2022;**234**(3):867–883. <https://doi.org/10.1111/nph.18029>
- Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J*. 1987;**6**(13):3901–3907. <https://doi.org/10.1002/j.1460-2075.1987.tb02730.x>
- Kloosterman B, De Koeyer D, Griffiths R, Flinn B, Steuernagel B, Scholz U, Sonnewald S, Sonnewald U, Bryan GJ, Prat S, et al. Genes driving potato tuber initiation and growth: identification based on transcriptional changes using the POCI array. *Funct Integr Genom*. 2008;**8**(4):329–340. <https://doi.org/10.1007/s10142-008-0083-x>
- Kloosterman B, Navarro C, Bijsterbosch G, Lange T, Prat S, Visser RGF, Bachem CWB. StGA2ox1 is induced prior to stolon swelling and controls GA levels during potato tuber development. *Plant J*. 2007;**52**(2):362–373. <https://doi.org/10.1111/j.1365-313X.2007.03245.x>
- Kondhare KR, Kumar A, Patil NS, Malankar NN, Saha K, Banerjee AK. Development of aerial and belowground tubers in potato is governed by photoperiod and epigenetic mechanism. *Plant Physiol*. 2021;**187**(3):1071–1086. <https://doi.org/10.1093/plphys/kiab409>
- Kondhare KR, Malankar NN, Devani RS, Banerjee AK. Genome-wide transcriptome analysis reveals small RNA profiles involved in early stages of stolon-to-tuber transitions in potato under photoperiodic conditions. *BMC Plant Biol*. 2018;**18**(1):284. <https://doi.org/10.1186/s12870-018-1501-4>
- Kondhare KR, Natarajan B, Banerjee AK. Molecular signals that govern tuber development in potato. *Int J Dev Biol*. 2020;**64**(1–2–3):133–140. <https://doi.org/10.1387/ijdb.190132ab>
- Kou E, Huang X, Zhu Y, Su W, Liu H, Sun G, Chen R, Yanwei H, Song S. Crosstalk between auxin and gibberellin during stalk elongation in flowering Chinese cabbage. *Sci Rep*. 2021;**11**(1):3976. <https://doi.org/10.1038/s41598-021-83519-z>
- Kundu A, Mishra S, Kundu P, Jogawat A, Vadassery J. Piriformospora indica recruits host-derived putrescine for growth promotion in plants. *Plant Physiol*. 2022;**177**(2):476–489. <https://doi.org/10.1093/plphys/kiab536>
- Lin T, Sharma P, Gonzalez DH, Viola IL, Hannapel DJ. The impact of the long-distance transport of a BEL1-like messenger RNA on

- development. *Plant Physiol.* 2013;**161**(2):760–772. <https://doi.org/10.1104/pp.112.209429>
- Liu Y, Ke L, Wu G, Xu Y, Wu X, Xia R, Deng X, Xu Q. mir3954 is a trigger of phase RNA s that affects flowering time in citrus. *Plant J.* 2017;**92**(2):263–275. <https://doi.org/10.1111/tpj.13650>
- Liu Y, Teng C, Xia R, Meyers BC. PhasiRNAs in plants: their biogenesis, genetic sources, and roles in stress responses, development, and reproduction. *Plant Cell.* 2020;**32**(10):3059–3080. <https://doi.org/10.1105/tpc.20.00335>
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−ΔΔCT} method. *Methods.* 2001;**25**(4):402–408. <https://doi.org/10.1006/meth.2001.1262>
- Ma W, Chen C, Liu Y, Zeng M, Meyers BC, Li J, Xia R. Coupling of micro RNA-directed phased small interfering RNA generation from long noncoding genes with alternative splicing and alternative polyadenylation in small RNA-mediated gene silencing. *New Phytol.* 2018;**217**(4):1535–1550. <https://doi.org/10.1111/nph.14934>
- Mahajan A, Bhogale S, Kang IH, Hannapel DJ, Banerjee AK. The mRNA of a Knotted1-like transcription factor of potato is phloem mobile. *Plant Mol Biol.* 2012;**79**(6):595–608. <http://dx.doi.org/10.1007/s11103-012-9931-0>
- Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant.* 1962;**15**(3):473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Nagasaki H, Itoh JI, Hayashi K, Hibara KI, Satoh-Nagasawa N, Nosaka M, Mukouhata M, Ashikari M, Kitano H, Matsuoka M, et al. The small interfering RNA production pathway is required for shoot meristem initiation in rice. *Proc Natl Acad Sci U S A.* 2007;**104**(37):14867–14871. <https://doi.org/10.1073/pnas.0704339104>
- Nakata M, Ohme-Takagi M. Quantification of anthocyanin content. *Bio-Protoc.* 2014;**4**(7):1428–1440. <https://doi.org/10.21769/BioProtoc.1098>
- Navarro C, Abelenda JA, Cruz-Oró E, Cuéllar CA, Tamaki S, Silva J, Shimamoto K, Prat S. Control of flowering and storage organ formation in potato by FLOWERING LOCUS T. *Nature.* 2011;**478**(7367):119–122. <http://dx.doi.org/10.1038/nature10431>
- Nogueira FT, Madi S, Chitwood DH, Juarez MT, Timmermans MC. Two small regulatory RNAs establish opposing fates of a developmental axis. *Genes Dev.* 2007;**21**(7):750–755. <https://doi.org/10.1101/gad.1528607>
- Rock CD. Trans-acting small interfering RNA4: key to nutraceutical synthesis in grape development. *Trends Plant Sci.* 2013;**18**(11):601–610. <https://doi.org/10.1016/j.tplants.2013.07.006>
- Rogers K, Chen X. Biogenesis, turnover, and mode of action of plant microRNAs. *Plant Cell.* 2013;**25**(7):2383–2399. <https://doi.org/10.1105/tpc.113.113159>
- Roumeliotis E, Kloosterman B, Oortwijn M, Kohlen W, Bouwmeester HJ, Visser RG, Bachem CW. The effects of auxin and strigolactones on tuber initiation and stolon architecture in potato. *J Exp Bot.* 2012a;**63**(12):4539–4547. <https://doi.org/10.1093/jxb/ers132>
- Roumeliotis E, Kloosterman B, Oortwijn M, Lange T, Visser RG, Bachem CW. Down regulation of StGA3ox genes in potato results in altered GA content and affect plant and tuber growth characteristics. *J Plant Physiol.* 2013a;**170**(14):1228–1234. <https://doi.org/10.1016/j.jplph.2013.04.003>
- Roumeliotis E, Kloosterman B, Oortwijn M, Visser RG, Bachem CW. The PIN family of proteins in potato and their putative role in tuberization. *Front Plant Sci.* 2013b;**4**:524. <https://doi.org/10.3389/fpls.2013.00524>
- Roumeliotis E, Visser RG, Bachem CW. A crosstalk of auxin and GA during tuber development. *Plant Signal Behav.* 2012b;**7**(10):1360–1363. <https://doi.org/10.4161/psb.21515>
- Sanz MJ, Mingo-Castel A, van Lammeren AA, Vreugdenhil D. Changes in the microtubular cytoskeleton precede in vitro tuber formation in potato. *Protoplasma.* 1996;**191**(1–2):46–54. <https://doi.org/10.1007/BF01280824>
- Schwach F, Vaistij FE, Jones L, Baulcombe DC. An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. *Plant Physiol.* 2005;**138**(4):1842–1852. <http://dx.doi.org/10.1104/pp.105.063537>
- Shaikh M, Bahuliker R, Chitnis A, Pandit S. GA3-mediated reforestation pioneering mechanism of actinorhizal *Elaeagnus conferta* Roxb. in the slashed and burnt shifting cultivation lands in India's Megadiversity hotspot. *Restor Ecol.* 2022;**31**(3):e13705. <https://doi.org/10.1111/rec.13705>
- Sharma P, Lin T, Hannapel DJ. Targets of the StBEL5 transcription factor include the FT ortholog StSP6A. *Plant Physiol.* 2016;**170**(1):310–324. <https://doi.org/10.1104/pp.15.01314>
- Shibaoka H. Regulation by gibberellins of the orientation of cortical microtubules in plant cells. *Funct Plant Biol.* 1993;**20**(5):461–470. <https://doi.org/10.1071/PP9930461>
- Shivaprasad PV, Chen HM, Patel K, Bond DM, Santos BA, Baulcombe DC. MicroRNA superfamily regulates nucleotide binding site–leucine-rich repeats and other mRNAs. *Plant Cell.* 2012;**24**(3):859–874. <http://dx.doi.org/10.1105/tpc.111.095380>
- Šimura J, Antoniadis I, Široká J, Tarkowská D, Strnad M, Ljung K, Novák O. Plant hormonomics: multiple phytohormone profiling by targeted metabolomics. *Plant Physiol.* 2018;**177**(2):476–489. <https://doi.org/10.1104/pp.18.00293>
- Tirumalai V, Swetha C, Nair A, Pandit A, Shivaprasad PV. mir828 and miR858 regulate VvMYB114 to promote anthocyanin and flavonol accumulation in grapes. *J Exp Bot.* 2019;**70**(18):4775–4792. <https://doi.org/10.1093/jxb/erz264>
- Varkonyi-Gasic E, Wu R, Wood M, Walton EF, Hellens RP. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant Methods.* 2007;**3**(1):1–12. <https://doi.org/10.1186/1746-4811-3-12>
- Xia R, Xu J, Meyers BC. The emergence, evolution, and diversification of the miR390-TAS3-ARF pathway in land plants. *Plant Cell.* 2017;**29**(6):1232–1247. <https://doi.org/10.1105/tpc.17.00185>
- Xia R, Zhu H, An YQ, Beers EP, Liu Z. Apple miRNAs and tasiRNAs with novel regulatory networks. *Genome Biol.* 2012;**13**(6):1–18. <https://doi.org/10.1186/gb-2012-13-6-r47>
- Xu X, van Lammeren AAM, Vermeer E, Vreugdenhil D. The role of gibberellin abscisic acid and sucrose in the regulation of potato tuber formation in vitro. *Plant Physiol.* 1998a;**117**(2):575–584. <https://doi.org/10.1104/pp.117.2.575>
- Xu X, Vreugdenhil D, van Lammeren AAM. Cell division and cell enlargement during potato tuber formation. *J Exp Bot.* 1998b;**49**(320):573–582. <https://doi.org/10.1093/jxb/49.320.573>
- Yoshikawa M, Peragine A, Park MY, Poethig RS. A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. *Genes Dev.* 2005;**19**(18):2164–2175. <https://doi.org/10.1101/gad.1352605>
- Zhai J, Jeong DH, De Paoli E, Park S, Rosen BD, Li Y, González AJ, Yan Z, Kitto SL, Grusak MA, et al. MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs. *Genes & Development* 2011;**25**(23):2540–2553. <http://dx.doi.org/10.1101/gad.177527.11>
- Zhang YC, Lei MQ, Zhou YF, Yang YW, Lian JP, Yu Y, Feng YZ, Zhou K, He RR, He H, et al. Reproductive phasiRNAs regulate reprogramming of gene expression and meiotic progression in rice. *Nat Commun.* 2020;**11**(1):1–11. <https://doi.org/10.1038/s41467-020-19922-3>

Curriculum-Vitae

Ms. Nilam Namdeo Malankar

Integrated Ph.D. student

Biology Division,
Indian Institute of Science Education and Research (IISER) Pune
Maharashtra – 411008, India

Phone No. +91-9773182919

E-mail: nilam.malankar@students.iiserpune.ac.in,
malankar.nilam@gmail.com



Educational Qualifications

Degree	Year	University/Board	College/School	CGPA
Ph.D. in Plant Molecular Biology	2016-present	Indian Institute of Science Education and Research (IISER) Pune	IISER Pune	-NA-
M.Sc. Biology	2016	Indian Institute of Science Education and Research (IISER) Pune	IISER Pune	8.3
B.Sc. Biotechnology	2014	Mumbai University	Ramnarain Ruia college, Mumbai	9.4
H.S.C.	2011	Maharashtra state board	Ramnivas Ruia college, Mumbai	89.83%
S.S.C	2009	Maharashtra state board	MCM girls high school, Mumbai	92.76%

Research Interests

Understanding the molecular dynamics of stolon-to-tuber transitions in potato development.

Publications

1. **Malankar N**, Kondhare KR, Saha K, Mantri M, Banerjee AK (2023) The phased short-interfering RNA siRD29(-) regulates *GIBBERELLIN 3-OXIDASE 3* during stolon-to-tuber transitions in potato. **Plant Physiology**, [DOI:10.1093/plphys/kiad493](https://doi.org/10.1093/plphys/kiad493).

2. Kondhare KR, Kumar A, Patil NS, **Malankar N**, Saha K, Banerjee AK (2021) Development of aerial and belowground tubers in potato is governed by photoperiod and epigenetic mechanism. **Plant Physiology**, November 2021, Vol. 187 (3), 1071–1086 DOI: [DOI:10.1093/plphys/kiab409](https://doi.org/10.1093/plphys/kiab409).
3. Kumar A, Kondhare KR, **Malankar N**, Banerjee AK (2020) A Polycomb group methyltransferase StE(z)2 and deposition of H3K27me3 and H3K4me3 regulate the expression of tuberization genes in potato. **Journal of Experimental Botany**, February 2021, Vol. 72 (2), 426–444 DOI: [10.1093/jxb/eraa468](https://doi.org/10.1093/jxb/eraa468).
4. Kondhare KR, **Malankar N**, Devani RS, Banerjee AK (2018) Genome-wide transcriptome analysis reveals small RNA profiles involved in early stages of stolon-to-tuber transitions in potato under photoperiodic conditions. **BMC Plant Biology**, Vol 18, 284 DOI: [10.1186/s12870-018-1501-4](https://doi.org/10.1186/s12870-018-1501-4).
5. Natarajan B, Kalsi H, Godbole P, **Malankar N**, Thiagarayaselvam A, Siddappa S, Thulasiram HV, Chakraborti S, Banerjee AK (2018) MiRNA160 is associated with local defense and systemic acquired resistance of potato during Phytophthora infestans infection. **Journal of Experimental Botany**, April 2018, Vol. 69 (8), 2023–2036 DOI: [10.1093/jxb/ery025](https://doi.org/10.1093/jxb/ery025).

Teaching Experience

- Teaching Assistantship (TA) for BS-MS students at IISER Pune.
[Introductory Biology-1 (BIO-101) and Introductory Biology-2 (BIO-102)]
- Supervision of 1 MS project for BS-MS student and 3 students with summer internships.

Research Skills

- Routine Molecular biology techniques.
- Plant tissue culture and transformations with potato and tobacco plants.
- Bioinformatics and Computation: Small RNA sequencing analysis.
- Small RNA related work: Cleavage site mapping and cloning.
- DNA-Protein Interaction Techniques
- LC-MS/MS-based plant hormone quantifications.

Conference Presentations

- Delivered an Oral presentation on PhasiRNA siRD29(-) regulates gibberellin biosynthesis gene (StGA3ox3) to mediate stolon-to-tuber transitions in potato development (**Plant Biology 2023**) at Savannah, GA, USA.
- Presented a poster titled “PhasiRNA siRD29(-) targets a gibberellin biosynthesis gene (StGA3ox3) to regulate stolon-to-tuber transitions of potato development” at “Current Trends

and Future Prospects of Plant Biology” (**CTFPPB 2023**) held at the School of Life Science, University of Hyderabad, India

- Presented a poster titled “Photoperiod dependent small RNA landscape during stolon to tuber transition stages of potato” at International Conference on Molecular Signaling (**ICMS-2019**) at Department of Zoology, SPPU and NCCS Pune.
- Presented a poster titled “Photoperiod dependent miRNA landscape during stolon to tuber transition stages of potato at “International Conference on Plant Developmental Biology & 3rd National Arabidopsis Meeting (**ICPDB-2017**)” at NISER Bhubaneswar.
- Attended “Biennial Meeting of Indian Society of Developmental Biology (**InSDB-2017**)” at IISER Pune.

Awards and achievements

- ASPB travel award 2023 by American Society of Plant biologist’s to attend the Plant Biology 2023 conference.
- Ekalavya travel award 2023 by Ignite Life science foundation to attend the Plant Biology 2023 conference.
- Senior Research Fellowship, 2019-2022, by Council of Scientific & Industrial Research (CSIR), Government of India.
- Junior Research Fellowship, 2017-2018, by Council of Scientific & Industrial Research (CSIR), Government of India.
- Dr. Homi Bhabha junior scientist award, 2008, by MSTA (Mumbai Science Teacher’s Association)

References

Prof. Anjan K. Banerjee

Professor
Biology Division,
IISER Pune, Pune, India-411008
E-mail: akb@iiserpune.ac.in
<http://sites.iiserpune.ac.in/~akb/>

Dr. Krishanpal Karmodiya

Assistant Professor
Biology Division,
IISER Pune, Pune, India-411008
E-mail: krish@iiserpune.ac.in
<https://krishanpalk.wixsite.com/krish>