NOTE TO USERS

This reproduction is the best copy available.

UMI[®]

The Growth of the Vascular Meristem is Negatively Regulated by CLV1 in *Arabidopsis*

By

Shuyou Han

**A thesis submitted to the Faculty of Graduated Studies and Research
In partial fulfillment of the requirements for the degree of
Master of Science**

**Ottawa –Carlton Institute of Biology
Carleton University
Ottawa, Ontario
Canada**

**April 2004
©(Shuyou Han) 2004**

National Library
of Canada

Bibliothèque nationale
du Canada

Acquisitions and
Bibliographic Services

Acquisitions et
services bibliographiques

395 Wellington Street
Ottawa ON K1A 0N4
Canada

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

ISBN: 0-612-93981-2

Our file Notre référence

ISBN: 0-612-93981-2

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this dissertation.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de ce manuscrit.

While these forms may be included in the document page count, their removal does not represent any loss of content from the dissertation.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

PAGINATION ERROR.

TEXT COMPLETE.

ERREUR DE PAGINATION.

LE TEXTE EST COMPLET.

Abstract

The shoot apical meristem produces all above-ground post-embryonic organs in higher plants, such as leaves and stems, as well as the primary vasculature. Secondary xylem and phloem is produced by another meristematic tissue known as the vascular cambium. It has previously been shown that the growth of the shoot and the floral meristems are negatively regulated by the CLV1 protein, such that the *clv1* mutant of *Arabidopsis* has unusually large floral and shoot apical meristems. In this study, we have investigated whether *CLV1* also plays a role in the regulation of the vascular cambium.

To produce a *clv1* mutant in *Arabidopsis*, we have down-regulated *CLV1* expression by transforming *Arabidopsis* with antisense constructs of *CLV1* gene. Out of 41 transgenic lines, 8 lines showed *clv1*-like phenotype, which have extra petals or deformed flower shape. Southern blots have been performed to confirm the number of inserted transgenes. Results of Northern blots showed that the antisensed genes were highly expressed, and expression level of endogenous *CLV1* gene was lowered by expression of antisense mRNA.

The homozygous transgenic plants and their wild type counterpart were grown for 4 months under short day conditions to induce the maximum amount of secondary growth. Compared to the wild type, the mutant lines produced much more xylem and phloem, suggesting that *CLV1* also negatively regulates the growth of the vascular meristem. The finding that *CLV1* is expressed in the two-month-old hypocotyl give us the first compelling evidence that *CLV1* is directly involved in the wood formation.

While this phenotype is interesting in *Arabidopsis*, it would be far more biotechnologically relevant if the same phenotype could be observed in a woody plant. The antisense construct of CLV1 homolog in poplar has been constructed, and further research work is under the way.

Acknowledgments

First of all, I would like to express my deep gratitude to my supervisor Dr. Sharon Regan for her patient guidance and encouragement during the development of this thesis, as well as the chance to attend several scientific conferences.

I would also like to thank my committee members, Dr. Myron Smith and Dr. Doug Johnson, for their helpful comments and suggestions throughout the course of this thesis.

I was also lucky to have the support of several professors from Department of Biology, in particular Dr. Bill Willmore, and Dr. Tim Xing.

Thanks also go to grad students Chris Kozela who gave me great help whenever available. The friends I made at Carleton University are the source of happiness and wisdom. I am grateful to all of them for their support during my stay in Ottawa.

Thanks to Carleton University and Genome Canada for financial support for this research.

Nothing is more important than my family's supports and encouragements. I could not finish my thesis without my wife's dedication.

Table of Contents

ABSTRACT.....	III
ACKNOWLEDGMENTS.....	V
LIST OF FIGURES	IX
LIST OF TABLES	X
ABBREVIATIONS.....	XI
1. INTRODUCTION.....	1
1.1 <i>ARABIDOPSIS</i> AS A MODEL PLANT	1
1.2 SHOOT APICAL MERISTEM STRUCTURE AND PHYSIOLOGY	3
1.3 A BRIEF REVIEW OF CLAVATA1 REGULATION STUDIES IN <i>ARABIDOPSIS</i>	9
1.4 <i>CLV1</i> GENE EXPRESSION	13
1.5 EXTRA PETALS AS THE INDICATOR OF A <i>CLV1</i> MUTANT	13
1.6 THE <i>CLV1</i> SIGNALING PATHWAY	14
1.7 CLAVATA AND WUSCHEL REGULATORY LOOP	19
1.8 SECONDARY GROWTH STUDIES IN NON-WOODY PLANT SPECIES	20
1.9 THE RATIONAL OF <i>CLV1</i> REGULATION OUTSIDE THE SAM.....	21
1.10 T-DNA IS GOOD STRATEGY FOR REVERSE GENETICS.....	22
1.11 TISSUE CULTURE-FREE PLANT TRANSFORMATION.....	24
1.12 THE MECHANISM OF <i>ARABIDOPSIS</i> TRANSFORMATION.....	26
1.13 ANTISENSE TECHNOLOGY IS GOOD WAY OF GENE SILENCING	27
1.14 THE OBJECTIVE.....	30
2. MATERIALS AND METHODS	31
2.1 SEED STERILIZATION AND VERNALIZATION	31
2.2 PLANT GROWING CONDITIONS	31
2.3 RNA ISOLATION FROM <i>ARABIDOPSIS</i>	32
2.4 AGAROSE GEL ELECTROPHORESIS	33
2.5 FIRST-STRAND cDNA SYNTHESIS.....	33
2.6 PRIMER DESIGN	34
2.6.1 Primers for Cloning.....	34
2.6.2 Primers for Making Probes.....	35
2.6.3 Primers for Confirming the Inserts.....	35
2.7 RT-PCR AMPLIFICATION OF DNA	36
2.8 ISOLATION OF PCR AMPLIFIED FRAGMENT.....	36
2.9 PREPARATION OF <i>E. COLI</i> COMPETENT CELLS	37
2.9.1 Electro Method.....	37
2.9.2 Chemical Method	39
2.10 PREPARATION OF <i>AGROBACTERIUM TUMEFACIENS</i> COMPETENT CELLS	39
2.11 CLONING PCR PRODUCTS USING pGEM-T® EASY VECTOR	40
2.11.1 Ligation of PCR Amplified Fragments into pGEM-T® Easy Vector... ..	40
2.11.2 Transformation of Plasmid DNA Into <i>E. coli</i>	43

2.11.3 Isolation of Recombinant Plasmids.....	43
2.12 RECOMBINANT PLASMID DIGESTED WITH <i>BAM</i> HI AND <i>KPM</i>	45
2.13 GENE CLEAN	45
2.14 DNA PRECIPITATION	46
2.15 pSOL2 VECTOR	46
2.16 THE PLANT TRANSFORMATION VECTOR--- pSOL6	50
2.17 TRANSFORMATION OF RECOMBINANT pSOL6 PLASMID INTO <i>AGROBACTERIUM</i>	53
2.18 SEQUENCING RECOMBINANT VECTOR	54
2.19 TRANSFORMATION OF <i>ARABIDOPSIS THALIANA</i>	54
2.20 SELECTION OF PUTATIVE TRANSFORMANTS USING AN ANTIBIOTIC MARKER	55
2.21 GENOMIC DNA ISOLATION FROM <i>ARABIDOPSIS</i>	56
2.22 SOUTHERN BLOT.....	57
2.23 NORTHERN BLOT	59
2.24 PREPARE THE HAND SECTION OF HYPOCOTYLS AND STEMS	62
3. RESULTS.....	64
3.1 CLONING OF <i>CLV1</i> INTO A PLANT TRANSFORMATION VECTOR.....	64
3.1.1 RNA Isolation from <i>Arabidopsis</i>	64
3.1.2 RT-PCR Results.....	64
3.1.3 Ligation of PCR products into pGEM®-T Easy Vector.....	64
3.1.4 Restriction Digestion to Confirm the Presence of an Insert	65
3.1.5 Insert Sequence Confirmation.....	65
3.1.6 Fragment Transfer from pGEM®-T Easy Vector to pSOL2	65
3.1.7 Restriction Digest of pSYH3 and pSYH4 to Release the Gene with the 35S Promoter Region.....	66
3.1.8 Fragment Transfer from pSOL2 to pSOL6.....	66
3.2 <i>ARABIDOPSIS</i> TRANSFORMATION	75
3.2.1 Flower-dip Transformation.....	75
3.2.2 Result of Transformation.....	75
3.3 GENETIC ANALYSIS OF TRANSGENIC PLANTS	78
3.3.1 Phenotypic Analysis of Transgenic Lines.....	78
3.3.2 Genetic Analysis --- Segregation of <i>T</i> ₂ plants.....	85
3.3.3 Homozygous Mutants.....	87
3.3.4 Morphological Analysis of Antisense Transformed <i>T</i> ₁ Plants vs. WT Plants.	87
3.4 MOLECULAR ANALYSIS OF <i>T</i> ₃ PROGENY	91
3.4.1 Confirmed Transgenes in Mutant Lines Using PCR.....	91
3.4.2 Southern Blot.....	91
3.4.3 RT-PCR to Detect the Expression of <i>CLV1</i>	95
3.4.4 Northern Blot.....	98
3.5 CELLULAR ANALYSIS OF <i>T</i> ₄ PROGENIES.....	101
3.5.1 Comparison of Radius of Xylem and Phloem/Cortex in Hypocotyl between WT and Mutant Lines	101
3.5.2. Comparison of Area (μm ²) of Xylem and Phloem/Cortex in Hypocotyl between WT and Mutant Lines	102

3.5.3 Comparison of Radius of Stem (mm) between WT and Mutant Lines	108
4. DISCUSSION	112
4.1. IDENTIFYING <i>CLV1</i> -LIKE PHENOTYPES IN MUTANT LINES	113
4.2. THE GROWTH OF VASCULAR MERISTEMS IS NEGATIVELY REGULATED BY <i>CLV1</i>	114
4.3. IS ANTISENSE THE BEST STRATEGY FOR DOWNREGULATING <i>CLV1</i> ?	117
4.4 TISSUE SPECIFIC EXPRESSION PROMOTER	119
4.5. FUTURE RESEARCH WORK.....	120
5. REFERENCES	122

List of Figures

Figure 1. 1 The schematic of longitudinal section of apical meristem of <i>Arabidopsis</i>	5
Figure 1. 2 The shoot apex of <i>Arabidopsis</i> is divided into three layers.....	7
Figure 1. 3 The schematic structure of CLV1.....	11
Figure 1. 4 Schematic of CLV signaling complex.	17
Figure 2. 1The map of pGEM-T [®] Easy vector.....	41
Figure 2. 2 The map of pSOL2 vector.	48
Figure 2. 3 The map of the plant transformation vector - pSOL6.....	51
Figure 3. 1 Agarose gel picture showing total RNA (A) and RT-PCR products...	67
Figure 3. 2The part of double strand sequence of <i>CLV1</i> in <i>Arabidopsis</i>	69
Figure 3. 3 Recombinant pGEM-T vector and digestion.	71
Figure 3. 4 The recombinant pSOL2 and pSOL6 plasmids.	73
Figure 3. 5 The appearance of the transformed seedlings.	76
Figure 3. 6The <i>CLV1</i> gene and protein sequence.....	80
Figure 3. 7 Comparison of WT and transgenic antisense flowers, floral meristem and siliques in the T ₂ generation.	83
Figure 3. 8 The comparisons of WT plants and T ₁ antisense transformed plants.	89
Figure 3. 9 Confirmation of transgenes in the transformed plants.....	93
Figure 3. 10 The expression pattern of <i>CLV1</i> gene.....	96
Figure 3. 11 Northern blot analysis.	99
Figure 3. 12 The transverse stem and hypocotyl sections of WT and antisensed transgenic plant.	104
Figure 3. 13 Comparison of xylem and phloem/cortex radius and area of cross section between WT and mutant lines.....	106
Figure 3. 14 Comparison of stem radius of cross section between WT and mutant lines.	110

List of Tables

Table 3. 1 Screening seeds of T ₂ transgenic plants on MS solid media with Kanamycin.	86
Table 3. 2 Average of each measurement of WT and T ₁ antisense transformed plant.....	87
Table 3. 3 Radius (μm) of Xylem and Phloem/Cortex in Hypocotyl between WT and Mutant Lines.....	101
Table 3. 4 Area (μm ²) of Xylem and Phloem/Cortex in Hypocotyl between WT and Mutant Lines.....	102
Table 3. 5 The radius (mm) of stems of WT and transgenic plants.....	108

Abbreviations

35S	Cauliflower mosaic virus 35S RNA
AMV	alfalfa mosaic virus enhancer
Amp	ampicillin
ATP	adenosine-5'-triphosphate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CLV	Clavata
CM	cambial meristem
cpm	counts per minute
D	day
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
dNTP	dinucleotide triphosphate
dTTP	deoxythymidine triphosphate
dH ₂ O	distilled water
ddH ₂ O	distilled de-ionized water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate of any the nucleic acid bases
DTT	dithiothreitol
E. coli	Escherichia coli
EDTA	ethylene-diamine-tetra-acetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N, N',N'-tetraacetic acid
EtBr	ethidium bromide
FM	floral meristem
HCl	hydrochloric acid
G	graviton
g	grams
hr	hour
IPTG	isopropyl β-D-thiogalactosidase
Kan	kanamycin
Kan 50	50mg/L kanamycin
Kan ^R	kanamycin resistant
Kan ^S	kanamycin sensitive
Kb	kilo-base
KCl	potassium chloride
kV	kilovolts
L	liter
LB	Luria broth

LER	Landsberg erecta
LRR	Leucine Rich Repeat
M	molar
mg	milligram
MgCl ₂	magnesium chloride
min	minute
mL	milliliter
mM	millimolar
mon	month
mRNA	messenger RNA
mv	molecular weight
NaCl	sodium chloride
ng	nanogram
nt	nucleotide
NaOAc	sodium acetate
NaOH	sodium hydroxide
OD	optical density
oligo dT	oligothymidylic acid
ORF	open reading frame
pHAN1	plamid Shuyou Han-1
pHAN2	plamid Shuyou Han-2
PCR	polymerase chain reaction
PEG	polyethylene glycol
QIA	QIAGEN®
RLK	receptor-ligand-kinase
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
rpm	revolution per minute
RT	room temperature
RT-PCR	reverse-transcriptase polymerase chain reaction
SAM	shoot apical meristem
SDS	sodium dodecyl sulfate
sec	second
SSC	standard sodium citrate
STM	shoot meristemless
TAE	Tris-acetate buffer
TE	10 mM Tris, 1mM EDTA (pH, 8.0)
T-DNA	transferred DNA
TENS	NaCl in TE buffer
Ti	Tumor Inducing
Tris	Tris (hydroxymethyl) aminomethane
tRNA	transfer RNA
U	unit
UV	ultra-violet
VC	vascular cambium

μg	microgram
μl	micro liter
μM	micro molar
vir	virulence region
X-Gal	5-bromo-4-choro-3-indolyl β-D-galactopyranoside
YEP	yeast extract and peptone media

1. Introduction

1.1 *Arabidopsis* as a Model Plant

Arabidopsis is a member of mustard family (Cruciferae or *Brassicacease*) with a broad natural distribution throughout Europe, Asia, and North America. There are many ecotypes available for experiment analysis. The Columbia and Landsberg ecotypes are accepted standards for genetic and molecular studies (Meinke *et al.*, 1998).

The reasons *Arabidopsis* has been a model system for both developmental genetics and molecular biology are due to its small size, short generation time, large progeny output of 10,000 seeds per plant, ease of transformation, wide variety of mutant strains and the completion of its genome sequence (Weigel *et al.*, 2002).

Almost everything about *Arabidopsis* is small. Flowers are 2 mm long and self-pollinate as the bud opens. Seeds are 0.5 mm in length at maturity. Seedlings develop into rosette plants that range from 2 to 10 cm in diameter. Plants can be grown in Petri plates or maintained in pots located either in a greenhouse or under fluorescent light in the laboratory. Flowers are composed of an outer whorl of four green sepals and inner whorls containing four white petals, six stamens and a central gynoecium that develops into the silique. Mature plants reach 15 to 20 cm in height.

In terms of the research history of *Arabidopsis*, the earliest non-taxonomic appearance of *Arabidopsis* in the literature of botany appears to be a paper by Alexander Braun in 1873, describing a mutant plant found in a field near Berlin

(Meyerowitz *et al.*, 2001). It was first chosen as a model genetic organism by Laibach in Europe and later studied in detail by Redei in the United States (Meinke *et al.*, 1998). The Modern era of *Arabidopsis* research began in 1987 with the opening of the Third International *Arabidopsis* Conference at Michigan State University and subsequent formation of an electronic *Arabidopsis* newsgroup.

There are a couple of the *Arabidopsis* research milestones listed below: 1) Meyerowitz *et al* summarized the reasons to adopt *Arabidopsis* as a model system for plant development, physiology, and molecular genetics in *Science* in 1985. 2) An *et al* (1986) first demonstrated that *Arabidopsis* could be transformed with exogenous DNA. 3) Chang *et al* (1990) published the first gene sequence in 1986 and An *et al* first established T-DNA-mediated transformation of *Arabidopsis*. 4) The first restriction fragment –length polymorphism map, T-DNA insertional cloning, map-based cloning and the extremely efficient vacuum infiltration method of transformation were carried out (Meyerowitz, 2001). 5) Complete and free sharing of experimental protocols and material was established as the norm, further motivating researchers to use the organism. The most recent methodological breakthrough is the completion of the DNA sequencing of *Arabidopsis* genome (The *Arabidopsis* Genome Initiative, 2000), thus forming a resource for future research.

Research with *Arabidopsis* has clarified the important role that analysis of the plant genome can play in understanding basic principles of biology relevant to a variety of species, including humans, since all eukaryotic organisms share a

common genetic ancestry. In a word, *Arabidopsis* can be a useful model not only for plant science, but also for whole organisms.

1.2 Shoot Apical Meristem Structure and Physiology

Embryogenesis in higher plants establishes a very simple structure that contains two stem cell populations, the shoot and root apical meristems (Clark, 2001a). In terms of the structural characteristics of the shoot apical meristem (SAM), it consists of three distinct radial domains (Cutter, 1965). Although cells in the zones do not have fixed fates, they usually follow predictable patterns. A small cluster of enlarged, highly vacuolated cells lie at the apex of SAM, and is termed the **central zone**. These cells divide infrequently relative to other cells in the SAM, and they are a source of cells for other regions and function as nonpermanent initials (Medford *et al.*, 1992). The cells in the central zone are surrounded by a **peripheral zone** made up of small, densely staining cells that divide more frequently than the cells in the central zone. They typically produce lateral appendages such as leaves. Beneath the central zone, in the deeper layers of the meristem, are columns of large vacuolated cell referred to as the **rib zone** (see Figure 1.1). These cells constitute the meristem pith and contribute to the bulk of the stem (Medford, *et al.*, 1992, Fletcher, 2002).

The SAM in most dicotyledonous flowering plants, including *Arabidopsis*, is also composed of three cell layers. The tunica consists of the L1 and L2 cell layers that remain clonally distinct, owing to anticlinal cell divisions, and that give rise to epidermal and mesophyll cells, respectively. The corpus, or L3 (see Figure

1.2), lies beneath the tunica layers and is characterized by more variable cell division planes and cell size. The corpus produces pith and **vascular tissue** (Clark *et al.*, 1997). The fact that the three cell layers are largely clonally isolated but coordinated in their growth and organ initiation indicates that cell-cell signaling might be important in SAM function (Meyerowitz, 1997). This is based on several findings. First, a small number of stem cells give rise to all the differentiated cell types of the adult plant, ruling out any important role for cell lineage patterns in regulating cell fate. Second, the organ initiated in the flanks of the meristem are composed of cells from all three clonally distinct layers (Jenik *et al.*, 2000), so these layers must communicate to execute organ formation in a coordinated fashion.

The stem cells are positioned at the center of the SAM, so that their growth and division leads to a continuous flow of progeny cells to the flanks of the meristem. Cells on the flanks of the meristem make a transition to a more differentiated fate and become competent to form organ primordia (Clark, 2001b). Both the renewal of precise number of stem cells and differentiation of many mature cell types from these cells depend on cell-cell communication and many researchers have focused on molecular mechanisms of cell-cell communication in the SAM. Some components in this signaling pathway have been uncovered by genetic analysis (Bowman *et al.*, 2000). It has been shown the main players genetically controlling the SAM are CLAVATA (CLV, including CLV1, CLV2 and CLV3) and WUSCHEL (WUS).

Figure 1. 1 The schematic of longitudinal section of apical meristem of *Arabidopsis*

CZ: central zone. PZ: peripheral zone. RZ: rib zone.

Figure 1. 2 The shoot apex of *Arabidopsis* is divided into three layers.

Layer 1 (L1) is a single layer of cells that generally only undergoes anticlinal divisions, and gives rise to the epidermis.

Layer 2 (L2) is also a single layer, and gives rise to ground tissue, while the innermost.

Layer (L3) forms the body of new tissues, including vascular and germline tissue.

1.3 A Brief Review of CLAVATA1 Regulation Studies in *Arabidopsis*

The SAM is responsible for aboveground organ initiation in higher plants, accomplishing continuous organogenesis by maintaining a pool of undifferentiated cells and directing descendant cells towards organ formation. Normally, proliferation and differentiation are balanced, so that the structure and size of the SAM is maintained (Clark *et al.*, 1997).

However, *Arabidopsis* plants homozygous for mutations at the *CLAVATA1* (*CLV1*) locus accumulate excess undifferentiated cells, suggesting that *CLV1* plays an important role in balancing the growth of the SAM.

The *CLV1* gene of *Arabidopsis* was first described in detail to affect floral meristem structure and floral organ number without affecting floral organ identity. *clv1* mutant plants have enlarged apical and floral meristems, leading to a variety of phenotypes including fasciation, disrupted phyllotaxis, and extra floral organs and whorls (Clark *et al.*, 1993). Floral meristems produced by the most severely affected *clv1* mutant can generate up to 7 or 8 carpels that fuse to form a club-shaped fruit. This phenotype gives the *clv1* mutants their name, from the Latin word *clavatus* meaning club-like (Fletcher *et al.*, 2002).

The first *clv* mutant, *clv1-4*, was produced by X-rays (Mckelvie, 1962); *clv1-1* is an ethyl methanesulfonate (EMS) – induced allele originally isolated in the ecotype Landsberg (Koorneef *et al.*, 1983). And Koorneef *et al.* were also the first researchers to map *CLV1* to chromosome 1. Leyser and Furrer (1992) had shown that *Arabidopsis* plants with mutations in *CLV1* could exhibit disrupted phyllotaxy, inflorescence fasciation, and altered leaf development and they

further demonstrated that *clv1* apical meristems were taller and wider than wild type, and three *clv1* alleles exhibited a variable number of extra organs in each whorl. A more significant finding is that *clv1* disrupts the apical meristem structure by enlarging it in weak alleles (*clv1-5*, 6, 7), causing fasciation in intermediate alleles (*clv1-1*), and causing massive over-proliferation in the strongest allele (*clv1-4*) (Clark *et al*, 1993).

In 1997, the *CLV1* gene was cloned. The *CLV1* transcript is predicted to be 3262 bases long and the *CLV1* genomic sequence contains a single 79-nucleotide intron at a position that is conserved in the leucine-rich repeat (LRR) kinase. Comparison of this sequence to the existing database suggested that *CLV1* encodes a putative receptor kinase, suggesting a role in signal transduction. The extracellular domain is composed of 21 tandem LRR that resemble LRR found in animal hormone receptors (see Figure 1.3). The LRR domain is followed by a stop-transfer sequence, suggesting a transmembrane domain. The putative intracellular domain contains all of the conserved residues found among serine/threonine protein kinases (Clark *et al.*, 1997).

There are several *Arabidopsis* expressed sequence tags (EST) that are similar to regions of CLV1 protein. These include LRR-containing sequences and kinase domain sequences, indicating that *Arabidopsis* genome contains many *CLV1*-like genes.

Figure 1. 3 The schematic structure of CLV1 protein.

Molecular cloning and expression pattern of the CLV1 gene showed that it encodes a receptor kinase, suggesting a role in signal transduction. The extracellular domain is composed of 21 tandem leucine-rich repeats.

1.4 *CLV1* Gene Expression

CLV1 mRNA is expressed specifically in a central region of the SAM and in early flower meristem. *In situ* RNA-hybridization experiments revealed that, in the inflorescence, *CLV1* is expressed in the shoot. *CLV1* mRNA is found in the presumptive SAM during embryo development, and is especially expressed in the L1 and L2 layers (Clark. 2001a). In the floral meristem, *CLV1* expression seemingly changes preceding organ initiation. For instance, *CLV1* expression is down regulated in the floral anlagen, and there is no expression in the stage 1 flowers, when flowers begin with the initiation of a floral buttress on the flank of the apical meristem (Smyth *et al.* 1990). Interestingly, the *CLV1* gene was found to be expressed in the pedicel (Dievart *et al.*, 2003). More research is needed to understand the expression of *CLV1* throughout plant development.

1.5 Extra Petals as the Indicator of a *clv1* Mutant

It has been shown that an over-proliferation of cells can result in a *clv1* SAM, growing over 1, 000-fold larger (by volume) than wild type (Clark *et al.*, 1997). The enlarged SAM in *clv1* is apparent as early as in the mature embryo (Running *et al.*, 1995). As the *clv1* mutant plants continue through vegetative and inflorescence development, their SAMs continue to enlarge by accumulation of undifferentiated cells. It is possible to detect the *clv1* phenotype by measuring the size of SAM, but because of the small size of *Arabidopsis* plants it is laborious and time consuming. The *CLV1* mutation has an additional effect on the development of the floral meristem that is much easier to detect than the effect

on the development of the SAM. Analysis of maturing flowers revealed that the *clv1* floral meristem continues to proliferate and give rise to a large mass of undifferentiated cells in the center of the flower, instead of terminating like the wild type floral meristem (Clark *et al.*, 1993). The safe conclusion can be drawn that *clv1* mutations affect the balance of cell proliferation versus cell differentiation.

This is consistent with the view that the floral primordium is derived from the SAM. This idea is further supported by genetic evidence, which suggests floral organs are modified leaves (Leyser *et al.*, 1992), so the numbers of petals are used to determine the extent of the *clv1*-like phenotype in the plants produced for this thesis.

1.6 The CLV1 Signaling Pathway

Clark *et al.* (1997) found that the *CLV1* gene encodes a putative receptor kinase, and its extracellular domain is composed of 21 tandem leucine-rich repeats, suggesting it is involved in signal transduction that acts in the communication of cell division and differentiation. But the regulatory mechanism has puzzled the researchers until the first model of CLV signaling mechanism had been proposed.

Trotochaud *et al.* (1999) found that CLV1 is in two protein complexes when it is purified from *Arabidopsis*. One is 185 kDa complex and the second is 450 kDa complex. An attractive interpretation is that the 185 kDa complex contains inactive CLV1, and the 450 kDa complex is composed of activated

CLV1 that is associated with downstream signaling proteins. Genetic studies are consistent with this hypothesis, for example, *clv1-10* extracts have no detectable 450 kDa complex, suggesting the formation of this complex depends on the kinase activity of CLV1, and the 450 kDa complex is very likely the active form of CLV1 (Clark, 2001a). CLV2 is similar to CLV1 in terms of the structure of the extracellular domain although it is not very similar at the level of primary sequence and CLV2 lacks a cytoplasmic domain (Jeong *et al.*, 1999). Genetic experiments established that CLV2 functions in the same pathway as CLV1 to regulate meristem development, and that CLV2 is required for the accumulation of CLV1. This raises the possibility that CLV1 and CLV2 directly bind to each other. Consistent with this, the mass of CLV1 (105 kDa) and CLV2 (80 kDa) add up to 185 kDa, indicating that inactive CLV1 complex might be a heterodimer of CLV1 and CLV2 (Clark, 2001a). The interesting thing is that an additional protein, CLV3, also acts in the same genetic pathway as CLV1 and it encodes a small-secreted protein that acts as a ligand for CLV1 (Fletcher *et al.*, 1999). Experiments showed that CLV1 is inactive without CLV3, suggesting CLV3 is required for the formation of active 450 kDa complex (Trotochaud, *et al.*, 1999). Overexpression of *CLV3* gives rise to an opposite phenotype to *clv1* or *clv3* mutant, that is, the SAM is reduced in size (Brand *et al.*, 2000). This is the first evidence that CLV3 works the upstream of CLV1.

The CLV1 450 kDa complex has a number of associated proteins, a subset of which is probably bound to phosphoserine residues in the kinase domain (Figure 3). A kinase-associated protein phosphatase (KAPP) was the first

protein identified to bind CLV1 (Williams *et al.*, 1997; Stone *et al.*, 1998). KAPP binds directly to CLV1 *in vitro* and is a component of 450 kDa complex *in vivo* (Trotochaud *et al.*, 1999). A hypothesis that KAPP negatively regulates CLV1 has been supported by two complimentary approaches. First, KAPP overexpression in the wild-type plants recreates a weak *clv1* phenotype . Second, when KAPP expression was suppressed in *clv1-1* plants, the mutant phenotype was suppressed, depending on the level of KAPP suppression (Stone *et al.*, 1998). The second known component of the 450-kDa CLV1 complex is Rho/Rac-GTPase-related protein (ROP) (Winge *et al.*, 2000). Using an antibody that cross-reacts with all of the *Arabidopsis* ROP isoforms, an appropriately sized cross-reacting protein was identified in the CLV1 450-kDa complex (Trotochaud *et al.*, 1999). Another interesting gene called POLTERGEIST (POL), which seems to function downstream as a negative regulator of CLV pathway, was also found (Yu *et al* 2000). Characterization of POL will certainly clarify the molecular events of the CLV1 pathway (Figure 1.4).

Figure 1. 4 Schematic of CLV signaling complex.

The CLV1 LRR-RLK (receptor-like kinase) forms a heteromeric complex with the CLV2 protein at the plasma membrane of interior SAM cells. The CLV3 multimer binds to the extracellular domain of CLV1/CLV2 heterodimer. The ligand binding of CLV3 drives CLV1 phosphorylation, and stimulate assembly of an active signaling complex that also contains a phosphatase (KAPP) and Rho-like GTPase (ROP). The signal is relayed from the cytosol to nucleus, potentially acting through a mitogen- activated protein kinase cascade (MAPK), to regulate *WUS* expression.

LRR, leucine repeat region; SS, disulphide bond. P, phosphorylation. KAPP, kinase-associated protein phosphatase. ROP, Rho-like GTPase. MAPK, mitogen-activated protein kinase. WUS, WUSCHEL.

1.7 CLAVATA and WUSCHEL Regulatory Loop

Schoof *et al* (2000) found that the stem cell population of the *Arabidopsis* SAM is maintained by a regulatory loop between the CLAVATA and WUSCHEL (WUS). The *WUS* gene encodes a putative homeodomain-containing transcription factor and because *wus* mutants lack stem cells at shoot apices it is believed to be involved in initiating stem cells at the SAM during embryogenesis, (Laux *et al.*, 1996; Mayer *et al.*, 1998). However, *WUS* is not expressed in stem cells, but in the cells underneath (Mayer *et al.*, 1998). So it is logical to assume that there might be a signal transduction pathway between CLV and WUS. Genetic interactions revealed that the CLV proteins regulate the expression of *WUS*, because *wus* mutations were largely epistatic to *clv* mutations, suggesting that WUS might act downstream of CLV and CLV negatively regulates *WUS* (Laux *et al.*, 1996). It has been shown that CLV3 is secreted from apical cells and diffuses to the most apical and lateral *CLV*-expressing cells to repress *WUS* expression (Brand *et al.*, 2000; Schoof *et al.*, 2000). Normally, *WUS*-expressing cells might not detect a significant amount of CLV3, because much of it is sequestered by the overlying *CLV1*-expressing cells (Trotochaud *et al.*, 2000; Lenhard *et al.*, 2003). Lenhard *et al* (2003) further provided the evidence that CLV3 protein functions as a mobile intercellular signal in the SAM that spreads laterally from the stem cells and acts both on their lateral neighbors and on the stem cells themselves to repress WUS transcription. They found that the spread and range of action of CLV3 could be limited by binding to its receptor CLV1. This regulated spread of a secreted signaling molecule enables the SAM to

permit the onset of cell differentiation in the periphery, but at same time to maintain a stable niche for its stem cells in the center.

1.8 Secondary Growth Studies in Non-woody Plant Species

The vascular cambium is a lateral meristem that is responsible for formation of secondary xylem (wood) and phloem. Significant advances towards understanding how the cambium meristem differentiates into secondary xylem require extensive studies from all levels of approach including histology, molecular biology, biochemistry, and genetics. Although cambial growth, including subsequent wood formation, has been successfully studied at different levels in many tree species (Han, 2001), the progress in the understanding of molecular and genetic controls regulating wood development has been slow. The major obstacles for functional genomics in trees are their large size, slow growth, large genome size, long generation times, and recalcitrance to biotechnological manipulation (Han, 2001). Therefore, it is valuable to have model system in which we can examine the genetic control of cambial growth at an accelerated pace.

The most used non-wood plant models are *Zinnia elegans* (Fukuda, 1996) and *Arabidopsis* (Lev-Yadun, 2001), and a number of genes associated with xylogenesis have been characterized in *Zinnia elegans* (Ye *et al.*, 1996) and *Arabidopsis* (Arioli *et al.*, 1998). The hypocotyl of *Arabidopsis* produces a relatively large amount of secondary vascular tissue when senescence is

delayed by the removal of inflorescences and when plants are grown at low population density (Lev-Yadun, 1997; Zhao et al., 2000).

Arabidopsis had previously been shown to undergo secondary growth (Gendreau *et al.*, 1997) and to have a vascular cambium (Busse *et al.*, 1999). The research on identity of the vascular cambium and the development of secondary xylem and phloem have not yet benefited much from the use of *Arabidopsis* as a genetic system until Chaffey *et al* (2002) investigated the usefulness of *Arabidopsis* as a model plant for studying wood formation and found that *Arabidopsis* undergoes extensive secondary growth in the hypocotyl, with the development of both a vascular and a cork cambium especially under short day conditions. In particular, extensive amounts of secondary xylem fibers and vessel elements are produced. During this second phase the secondary xylem of *Arabidopsis* closely resembles the anatomy of the wood of an angiosperm tree, and can be used to address basic questions about wood formation. This finding offers new prospects for the cambial growth and secondary xylem development. The secondary xylem in the hypocotyl of *Arabidopsis* will be investigated in this thesis as an indicator of the effect of CLV1 on wood formation.

1.9 The Rational of CLV1 Regulation Outside the SAM

It has been known that a mutation in *CLAVATA* (*CLV1*, *CLV2*, *CLV3*) loci lead to an enlarged SAM resulting from the accumulation of large pool of undifferentiated cells.

One important finding is that bundle number is proportional to meristem size. This could be a direct consequence of increase in meristem size or an indirect consequence of the increase in organ number associated with larger meristem (Doil *et al.*, 1998). And it has been found that strong *clv1* mutations show mature xylem elements in the meristem above where organ primordia are usually first seen. These findings suggest the *CLV1* could play a role in secondary growth or wood formation and they are consistent with our hypothesis that the growth of vascular cambium may also be negatively regulated by CLV1.

Another interesting finding is that the long-distance signaling in nodulation is directed by a *CLAVATA1*-like receptor kinase (Searle *et al.*, 2003). It has been known that proliferation of legume nodule primordia is controlled by shoot-root signaling known as autoregulation of nodulation (AON). Mutants defective in AON show super-nodulation and increased numbers of lateral roots. They demonstrated the AON in soybean is controlled by the receptor-like protein kinase GmNARK (*Glycine max* nodule autoregulation receptor kinase), which is similar to *Arabidopsis* CLV1. It has been shown that GmNARK in the shoot has a major role in long-distance communication between nodule and lateral root primordia. This finding gives us a clue that CLV1 not only might regulate the shoot and floral growth but also might regulate the growth of other organs.

1.10 T-DNA is Good Strategy for Reverse Genetics

The *Arabidopsis* genome has been sequenced and it appears to have approximately 30, 000 genes, and most of them have not been fully

characterized. The completion of the genome project, therefore, opens a new frontier for functional genomics. The accumulation of functional information about *Arabidopsis* genes will promote the application of reverse genetics methodologies to decipher the functions of novel genes that are homologous to known genes, or study unknown genes that interact biologically with genes of known function (Parinov *et al.*, 2000). The ability to move DNA into an organism and thereby alter its phenotype is central to both basic and applied molecular biology.

In terms of the history of plant transformation, researchers have been able to carry out controlled plant transformation with specific genes since 1983. The most common methods for introducing DNA into plant cells use *Agrobacterium tumefaciens* or rapidly propelled tungsten microprojectiles that have been coated with DNA (Hansen *et al.*, 1999). There are other methods, such as electroporation, microinjection, and delivery by virus (Bent, 2000).

Agrobacterium-mediated T-DNA transformation has been used for insertional mutagenesis in different plant species. Plant transformation by *Agrobacterium* results in the integration into nuclear genome of a sequence called the transfer DNA or T-DNA, which is carried on a bacterial plasmid. Large collections of T-DNA transformed *Arabidopsis* have been collected independently by several groups and have been used extensively for gene function studies (Krisan *et al.*, 1999).

There are a number of advantages of using T-DNA as an insertional mutagen. The first one is that it can directly generate stable insertions into

genomic DNA and does not require additional mutagen to stabilize the insert. The second advantage is that the T-DNA insertion appears to be completely random, as no T-DNA integration hot spot or integration preferences have been reported. However, some chromosomal rearrangements induced by T-DNA integration have been observed, leading to difficulties in genetic analysis of the insertion, such as mutant phenotypes that are not correlated with T-DNA insertion (Nacry *et al.*, 1998). Nevertheless, the development of highly efficient methods of T-DNA transformation for *Arabidopsis* has made it feasible for laboratories to generate transformants rapidly, and as a result T-DNA is now a widely used insertional mutagen in *Arabidopsis* (Clough *et al.*, 1998).

1.11 Tissue Culture-free Plant Transformation

Over the past decade, numerous *Agrobacterium*-mediated transformation systems have been developed in order to study gene expression, homologous recombination, or to functionally complement various *Arabidopsis* mutants (Clarke *et al.*, 1993). Although these systems can be used to produce large numbers of transformants, they all rely on tissue culture techniques. Tissue culture is labor intensive and can be difficult to master. In addition, even under optimal transformation and regeneration conditions tissues culture can result in somaclonal variation, morphological abnormalities, changes in chromosome number, and loss of fertility which are unrelated to the gene that is inserted (Velvekens *et al.*, 1988).

As an alternative to transformation procedures that employ *in vitro* regeneration of plants, Feldmann and Marks (1987) developed a non-tissue culture transformation protocol based on co-cultivation of germinating *Arabidopsis* seeds with *Agrobacterium*. This seed transformation method did not involve tedious and time-consuming tissues culture steps and it circumvented the problems associated with *in vitro* plant regeneration, but his particular technique did not have a high success rate and was not embraced by the *Arabidopsis* community as a viable alternative to traditional tissue culture techniques.

Another non-tissue culture *Arabidopsis* transformation approach named by “clip ‘n squirt” was described by Chang *et al.* (1990). Reproductive inflorescence shoots were clipped off, *Agrobacterium* was applied to center of the plant rosette, new inflorescences formed a few days later were again removed, *Agrobacterium* was reapplied and plants were then allowed to develop and set seeds. Transformants could be obtained and the method was more reliable than with seed treatment method. (Katavic *et al.*, 1994).

The most significant development in *Arabidopsis* transformation technologies was the “vacuum infiltration” method. *Arabidopsis* plants at early stages of flowering were uprooted and placed en masse into a bell jar in a solution of *Agrobacterium*. A vacuum was applied and then released. Plants were transplanted back to soil, grown to seed, and in the next generation stably transformed lines could be selected using the antibiotic appropriate for the selectable marker gene. This approach has proved successful in many laboratories and is now the method of choice for *Arabidopsis* transformation. A

modification of this technique, called flower-dip transformation was used in this project.

1.12 The Mechanism of *Arabidopsis* Transformation

The mechanism of *Arabidopsis* transformation underlying the “vacuum infiltration” transformation puzzled the researchers for years. It was shown early on that most primary transformants carry hemizygous T-DNA insertion events. The presence of the T-DNA on only one of two homologous chromosomes implies that productive transformation occurs late in floral development, after the divergence of male and female germ line (Bent, 2000). The fact that transformants carry the transgene in all parts of plant suggests that transformation occurs in developing flowers after individual gametophyte cell lineages form, but before extensive development of the embryo. To test where is the primary target for transformation, a couple of experiments have been done. Transformants were produced by outcrossing after *Agrobacterium*-inoculation of only the pollen donor or pollen recipient (Ye *et al.*, 1999). The results have shown no transformants were observed among the seeds produced following inoculation of pollen donor, but some transformants were recovered from seeds produced following inoculation of pollen recipient. And Ye *et al.* further showed that ovules are the primary target for transformation by GUS staining; *Agrobacterium* apparently enters *Arabidopsis* locules of ovules prior to their closure.

Another important finding is that the *Arabidopsis* transformation events appear to be independent even if they are from same plant or and in some cases even those from the same silique (Desfeux *et al.*, 2000). Thus the vacuum

infiltration transformation technique is a valuable method of producing a large number of independently transformed plants with stable insertions.

1.13 Antisense Technology is Good Way of Gene Silencing

One method to understand the function of a gene is to downregulate the expression of the gene by a variety of gene silencing techniques. The techniques can result in a loss-of-function phenotype that can lead to a better understanding of the gene's endogenous function in the plant.

One approach to understanding the function of gene is to introduce the antisense homolog of the gene into wild-type plants and study the effect of its expression on the phenotype of the plant. This is achieved through the production of transgenic plants in which the introduced gene is under the control of a specific promoter. The expression of antisense gene causes the down-regulation of gene expression due to an interaction between the RNA of endogenous gene and antisense RNA from the introduced gene (Rothstein *et al.*, 1987).

For a long time, RNA has been merely regarded as a molecule that can either function as a messenger (mRNA) or as part of the translation machinery (tRNA, rRNA). It has become clear that RNAs are versatile molecules that do not only play key roles in many important biological processes like splicing, editing, protein export and other, but also act catalytically like enzymes. Antisense RNA has proved to be a very useful tool in the gene function studies. Antisense RNAs

are small, diffusible, highly structured RNAs that act via sequence complementarity on target RNAs called sense RNAs.

The first natural antisense RNAs were discovered in 1981 independently in Tomizawas and Nordstroms laboratories (Brantl, 2002). These investigators found that small plasmid-encoded RNA regulators control the copy numbers of the *E. coli* plasmids ColE1 and R1 respectively (Brantl, 2002). In the classical case, antisense RNAs are encoded in cis, for instance, they are transcribed from a promoter located on the opposite strand of same DNA molecule, and are, therefore, fully complementary to their target RNAs. The antisense RNA can be complementary to different regions or the whole length of an mRNA. The length of antisense RNA is closely related to its function (Yin *et al.*, 2002). In the majority of cases, antisense RNA action entails posttranscriptional inhibition of target RNA function, that is, the RNA is made unavailable to the ribosome for translation.

Almost all naturally occurring antisense RNA regulated systems have been reported in prokaryotes, and only few systems are known from eukaryotes. In plants, no naturally occurring antisense RNAs have been found so far. However, artificially introduced antisense transcripts are believed to target mRNA for degradation. Here is a successful story of antisense RNA. As we know, most tomatoes that have to be shipped to market are harvested before they are ripe. Otherwise, ethylene synthesized by tomato causes them to ripen and spoil before they reach the customer. Transgenic tomatoes have been constructed, which carry a transgene that is transcribed into an antisense RNA

complementary to the mRNA for an enzyme involved in ethylene production. These tomatoes make only 10% of the normal amount of the enzyme. The significance of this work was to provide supermarket tomatoes with something closer to the appearance and taste of tomatoes harvested when ripe (Nagata *et al.*, 1995).

There are other two gene silencing techniques too. One is RNA interference (RNAi), also called post-transcriptional gene silencing or PTGS. RNAi means the ability of double-strand RNA (dsRNA) to suppress the expression of a gene corresponding to its own sequence. If the cell finds molecules of dsRNA, it uses an enzyme, which is named Dicer, to cut them into fragments containing 22 base pairs (also called small interfering RNAs or siRNA). Then two strands of each fragment separate and bind to the complementary sense sequence on a molecule of mRNA. This triggers cutting the corresponding mRNA, thus destroying its ability to be translated into a polypeptide (Wei *et al.*, 2001). Another gene silencing technique is called co-suppression. It means that introduction of extra copies of a given gene in a sense orientation can lead to suppression of expression of the introduced transgene and the corresponding endogenous gene. The working mechanism of co-suppression remains unknown.

The antisense technology will be employed to down-regulate the CLV1 gene expression level with hope to mimic the *clv1* phenotypes.

1.14 The Objective

Both antisense and cosuppression constructs of the *Arabidopsis CLV1* will be transformed into *Arabidopsis* using the flower-dip transformation method to ascertain whether an antisense or co-suppression strategy is sufficient to produce a *clv1*-like phenotype in homozygous lines. Should we be successful in altering the activity of the vascular cambium through the downregulation of *CLV1* by antisense or cosuppression, this will provide a proof of concept for the much longer-term goal of using the same strategy to alter the amount of wood formation in trees.

2. Materials and Methods

2.1 Seed Sterilization and Vernalization

Arabidopsis thaliana (L.) Heynh. Seeds of ecotype of Columbia were sterilized by soaking for eight min in a solution of 10% bleach and 0.02 % Triton X-100 followed by 2 min in ethanol with periodic shaking. After the solution was removed, the seeds were then washed in 10 to 12 changes of sterile distilled water. Seeds were stratified for 4 D at 4°C as this promotes uniform germination. Approximately 50 seeds were spotted on the surface of MS medium (4.33 MS salts, 30 g sucrose, 8 g agar per liter water, pH 5.7-5.8) in 100X20 mm Petri plates with a Pasteur pipette. The plates were sealed with parafilm and incubated under long day condition (22°C; 15 μ E/m²/s; 16 hr light/8 hr dark cycles) for 7-10 D.

2.2 Plant Growth Conditions

Arabidopsis thaliana (L.) Heynh seeds of ecotype Columbia were grown in an autoclaved mix of 80% sand and 20% Fisons Levington multi-purpose compost. Plants were typically planted 1 per 25cm² pot in moistened potting soil. The plants were maintained at 20-22 °C in growth chamber with florescent lighting at the same intensity as described above. The long day condition was setup as 16 hr light/8hr dark cycle light regimes, and the short day condition was setup 8 hr light /16hr dark cycle light regimes. Because *Arabidopsis* is a long day plant, short day conditions do not promote flowering and it results in extensive secondary growth.

2.3 RNA Isolation from *Arabidopsis*

Total RNA was isolated from whole *Arabidopsis thaliana* plants (Columbia strain) using the QIAGEN RNeasy Kit. Plant tissue was ground under liquid nitrogen to a fine powder using a mortar and pestle. Approximately 100 mg of ground tissue was transferred to a 1.5 mL microcentrifuge tube, with liquid nitrogen, and 450 µl of Buffer RLT (RNeasy Lysis Buffer), was immediately added. The mixture was vigorously vortexed. The lysate was then applied to the QIAshredder spin column that was sitting in a 2 ml collection tube. The column in the tube was spun for 2 min at maximum speed in a microcentrifuge. The flow-through in the collection tube was transferred into a new 1.5 ml microcentrifuge tube. To the isolated flow-through, 0.5 volumes of 100% ethanol was added and mixed by pipetting. The sample was transferred (including any precipitate) to an RNeasy mini spin column (included in kit) sitting in a 2 ml collection tube. The column in the tube was centrifuged for 15 sec at full speed in a microcentrifuge; the resulting flow through was discarded. 700 µl of Buffer RW1 was added to the column that was then incubated at room temperature for 5 min. The column and the collection tube were centrifuged for 15 sec at maximum speed in a microcentrifuge, flow-through and collection tube were discarded. The RNeasy column was transferred to a new 2 ml collection tube and 500 µl of Buffer RPE was added to the column. The column in the tube was centrifuged for 15 sec at maximum speed in a microcentrifuge, and the flow through was discarded. 500 µl of Buffer RPE was added into the column (in the collection tube) that was then centrifuged for 2 min at maximum speed in a

microcentrifuge to dry the RNeasy membrane. The column was then placed in a new 1.5 ml microcentrifuge tube and 30 to 50 µl of RNase-free water was added. The column in the tube was centrifuged for 1 min at maximum speed in a microcentrifuge. The flow through was collected (contains RNA). Agarose (1%) gel electrophoresis was used to confirm the presence of RNA. The remaining sample was stored at -80°C.

2.4 Agarose Gel Electrophoresis

All RNA and DNA samples were electrophoresed on 1% agarose gel in 1X TAE buffer (40 mM Tris-Acetate, 2 mM Na₂EDTA.2H₂O, pH, 8.5) with 0.15% ethidium bromide. Samples contained Orange G as a running dye, and were electrophoresed against 6 µl (0.5 µg) of 1 kb DNA ladder (Life Technology) (10 µl 1 kb DNA ladder (0.1 µg/µl), 90 µl 1X TE, 20 µl loading buffer) as a molecular weight marker. The agarose gel was electrophoresed on the Owl Separation System Model BIA (Owl Separation Systems Inc.) at 83 volts. The results were viewed and photographed using Gel-Doc (Alpha Innotech). For quantifying the amount of DNA in the sample, the intensity of 1.6 kb band of 1 kb ladder (50 ng) was visually compared with that of the sample DNA.

2.5 First-strand cDNA Synthesis

First strand complementary DNA (cDNA) was created from isolated *Arabidopsis* total RNA. To a fresh microtube was added 20 µl RNA, 5 µl 5X First strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, 50 mM

DDT), 2 µl (20 nM) dNTPs (Life Technology), 1 µl (50 nM) oligo dT primers (Life Technology), 2 µl (40 U) of reverse transcriptase (USB), and 1 µl (25U) of RNase inhibitor (USB). The reaction mixture was incubated at 37°C for 1 hr, and then stored at –20°C until needed.

2.6 Primer Design

2.6.1 Primers for Cloning

Primers were designed for *CLAVATA1* of *Arabidopsis* by the analysis of the known sequence in *Arabidopsis* available in [www. arabidopsis. org](http://www.arabidopsis.org). Forward and reverse primers were designed against this sequence. For the sense *CLV1* fragment, the *Bam*HI restriction site was added to the 5' end of the forward primer and the *Kpn*I restriction site was added to the 5' end of the reverse primer. For the antisense *CLV1* fragment, the *Kpn*I restriction site was added to the 5' end of the forward primer and the *Bam*HI restriction site was added to the 5' end of the reverse primer. Primers were synthesized by Invitrogen. The designed primers were as follows.

Sense:

Forward: 5' **GGA TCC** CCG GAT GCT CAC TGT TCT TTC TCC GGC G 3'

Reverse: 5' **GGT ACC** CTC TCC GGT GAG TGT ACA GCT CGC CA 3'

Antisense:

Forward: 5' **GGT ACC** CCG GAT GCT CAC TGT TCT TTC TCC GGC G 3'

Reverse: 5' **GGA TCC** CTC TCC GGT GAG TGT ACA GCT CGC CA 3'

Notes: The sequence in bold is the restriction site. The sequence of *Bam*HI is GGA TCC and the sequence of *Kpn*1 is GGT ACC.

2.6.2 Primers for Making Probes

To do Southern hybridization for checking the copy number of the transgene in the transformed plants, the probes were made by running PCR based on the primers against the 35S promoter region. The designed primers were as follows.

Forward: 5' GAG GCT TAC GCA GCA GGT CT 3'

Reverse: 5' CCA CGA TGC TCC TCG TGG GT 3'

To do northern hybridization to determine the expression level of *CLV1* gene both in wild type and transgenic plants, the probes were made by running PCR based on the primers against the cDNA sequence of *CLV1*. The designed primers were as follows.

Forward: 5' **GGA TCC** CCG GAT GCT CAC TGT TCT TTC TCC GGC G 3'

Reverse: 5' **GGT ACC** CTC TCC GGT GAG TGT ACA GCT CGC CA 3'

2.6.3 Primers for Confirming the Inserts

To double confirm the sense and antisense of cloned *CLV1* DNA fragments were in the right orientation and linked to 35S promoter region, the probes were designed against the 35S promoter region and NOS region of pSOL2 vector. The designed primers were as follows.

Forward: 5' TCC GGA AAC CTC CTC GGA TTC CAT TGC 3'

Reverse: 5' GAT AAT CAT CGC AAG ACC GGC AAC AGG 3'

2.7 RT-PCR Amplification of DNA

cDNA prepared from *Arabidopsis* total RNA was used for PCR. The primers used were described to amplify the coding region of the putative *CLV1* (605 bp). The PCR mixture was made to a final volume of 20 µl with 2 µl of 10x PCR Buffer, 0.4 µl of 10 mM dNTPs, 0.6 µl of 50 mM MgCl₂, 1µl of each 10 µM primer, 0.2 µl of 5 u/µl Taq polymerase, 5 µl of cDNA template, and 9.8 µl of sterile distilled water (to make the final volume to 20 µl). The PCR cycle used to amplify the gene with these primers is as follows: the PCR was set to 30 cycles with 94°C denaturing temperature for 30 sec, 64°C annealing temperature for 30 sec, and 72°C elongation temperature for 1 min. 1% agarose gel electrophoresis was used to confirm amplification of *CLV1*. Remaining PCR products were stored at 4°C until needed (long term storage at -20°C).

2.8 Isolation of PCR Amplified Fragment

PCR products were separated by 1% agarose gel electrophoresis, run at 83 volts for approximately 50 min. The putative amplified fragment was isolated from the gel using the GeneClean BIO 101 kit (Q. BIO gene) – all reagents were supplied with the kit. The area of gel containing the band of interest was cut from the gel and weighed. Three volumes of sodium iodide (included in kit) was added to the piece of gel in a 1.5 ml tube. The tube was incubated at 55°C for approximately 5 min, with occasional mixing, until the agarose had melted. Approximately 5 µl

suspended Glassmilk was added. The solution was incubated on ice for 5 min. The silica was then pelleted by spinning at full speed in a microcentrifuge for 5 sec, the supernatant was discarded. The pellet was resuspended in 400 µl New Wash (50 mM NaCl, 10 mM Tris-Cl, pH 7.5, 2.5 mM EDTA, 50 % ethanol) and then spun at full speed in a microcentrifuge for 5 sec, the supernatant was discarded. The pellet was then washed three times with New Wash buffer. After the supernatant from the 3rd wash had been removed the pellet was spun again for 5 sec to draw out any remaining buffer. The pellet was dried by leaving the tube open at 65 °C on the heatblock for 5 min to make sure all the ethanol had been removed. DNA was eluted from the glass milk by resuspending the pellet in approximately 10 µl of distilled, sterile water, centrifuging for 30 sec and then transferring the supernatant to a new 1.5 ml sterile tube. 1 % agarose gel electrophoresis (80 volts) was used to confirm presence of the isolated fragment. Remaining DNA was stored at –20°C until needed

2.9 Preparation of *E. coli* Competent Cells

2.9.1 Electro Method

E. coli competent cells were prepared as follows; a single colony of DH5α *E.coli* was used to inoculate 5 ml of LB (10g tryptone, 5 g yeast extract, 5 g NaCl in 1 L of distilled water), and grown overnight at 37°C with shaking (300 rpm). From the culture, 2.5 ml was used to inoculate 500 ml LB medium in a sterile 2 L flask, and grown at 37°C with shaking (300 rpm) to an optical density OD₆₀₀ of 0.6. Cells were chilled in an ice water bath for 10 min before being transferred to

a prechilled 500 ml centrifuge bottle. Cells were centrifuged at 4200 rpm for 20 min in a Sorvall RC 5C PLUS with a SLA-1500 rotor. The supernatant was poured off, and the pellet was resuspended in 5 ml ice-cold sterile water. An additional 500 ml of ice-cold sterile water was added and the solution was mixed, and centrifuged as above. The supernatant was poured off, and pellet was resuspended in 500 ml ice-cold sterile water, mixed and centrifuged as above. The supernatant was poured off immediately and the pellet was resuspended in approximately 2 ml of water. Then, the resuspended pellet was transferred to a sterile 50 ml polypropylene tube, and re-centrifuged as above. 40 ml of ice-cold sterile 10% glycerol was added to the cell suspension, and the cells were re-suspended. Cells were centrifuged for 10 min at 4,200 rpm in a SS-34 rotor in a Sorvall RC 5C PLUS centrifuge at 2 °C. The supernatant was discarded, the volume of pellet was estimated, and equal volume of 10% glycerol was added, the cells were re-suspended. The suspension was aliquoted into 100 µl volumes, frozen on dry ice, and stored at –80 °C until further use (Ausubel *et al.*, 2000).

For electroporation, DNA (less than 5 ng) was mixed with 40 µl of host cell and transferred into a 0.2 cm cuvette on ice. Electroporation was done with the following parameters: 2500V, 200 Ohms, and 25 F. After electroporation, 950 µl of SOC (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄) was added immediately and cells were incubated at 37 °C for 1 hr before plating to appropriate LB plates.

2.9.2 Chemical Method

Chemical transformation competent cells were prepared as follows. A single colony of DH5 α *E.coli* from a fresh plate culture was inoculated into 2-3 ml of LB broth (10g tryptone, 5 g yeast extract, 5 g NaCl in 1 L of distilled water, pH 7.5) and grown overnight at 37 °C with shaking. 1 ml of this culture was added to 100 ml LB broth and grown at 37 °C (around 90-120 min) until the OD₆₀₀ reached 0.6. Cells were then transferred into a cold centrifuge bottle and kept on ice for 10 min, and then centrifuged at 4000X g in GSA rotor for 5 min at 4 °C. The supernatant was discarded and the pellet was suspended in 33 ml of cold buffer (pH 5.8) which contained 100 mM RbCl, 50 mM MnCl₂.4 H₂O, 30 mM potassium acetate, 10 mM CaCl₂.2H₂O and 15% (w/v) glycerol. Cells were kept on ice for 15 min, and then centrifuged again at 4000X for 5 min. The pelleted cells were resuspended in 8 ml of cold buffer (pH 6.8) which contained 10 mM MOPS, 10 mM RbCl, 75 mM CaCl₂.2H₂O , and 15% (w/v) glycerol, and kept on ice for 15 min before being dispensed into cold microfuge tubes (200 μ l aliquots) and put them -70 °C freezer for later use.

2.10 Preparation of *Agrobacterium tumefaciens* Competent Cells

A single colony of *Agrobacterium tumefaciens* was used to inoculate 5 ml of YENB (0.75 % Bacto yeast extract, 0.8 % Bacto Nutrient Broth) and grown at 28 °C with shaking at 300 rpm, A 1L flask of YENB was inoculated with 5 ml culture and grown at 28 °C with shaking at 400 rpm to an optical density of 0.6. Cells were chilled in an ice-water bath for 5 min before being transferred to a

sterile pre-chilled 1 L centrifuge bottle. Cells were centrifuged and treated as aforementioned for *E. coli* culture.

2.11 Cloning PCR Products Using pGEM-T[®] Easy Vector

2.11.1 Ligation of PCR Amplified Fragments into pGEM-T[®] Easy Vector

The PCR fragment isolated from PCR was ligated into the 3015 base pair long pGEM-T[®] Easy vector (Promega) according to manufacturer's instruction. This vector has 3' poly-T tails that ligate to the poly A tails of PCR fragments. The map of pGEM-T Easy vector is attached here (Figure 2.1). The ligation was performed following the Promega standards, a 1:3 ratio (concentration) of vector: insert was generally used to maximize ligations. A typical ligation reaction included 5 µl of 2x Rapid Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM MgCl₂, 20 mM DTT, 1mM ATP 10% PEG, Promega), 1 µl of pGEM-T easy vector (50 ng) 1 µl of T4 DNA ligase (3 Weiss units/µl), 2 µl of PCR product, 1 µl of sterile distilled water. The ligation reaction was mixed by pipetting and incubated overnight at 4°C. The ligated DNA was stored at -20°C until needed for transformation.

Figure 2. 1 The map of pGEM-T[®]Easy vector.

(From [www. Promrga.com](http://www.Promrga.com))

2.11.2 Transformation of Plasmid DNA Into *E. coli*

Plasmid DNA (about 0.5 µg) was mixed with 50 µl ice-cold competent DH5 *E.coli* cells and placed in an *E. coli* Pulser (BIO-RAD) electroporation apparatus. Electroporation was carried out with a 1.8 kV with pulse controller at 200 ohms. Following electroporation, 1 mL of SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄) was added to the electroporated cells, and the mixture was incubated at 37 °C for one hour. 200 µl of re-suspended cells were plated on fresh LB plates (7.5 g agar per 500 ml Lb broth) containing 100 µg/mL Ampicillin, 0.5 mM IPTG, and 80 µg/mL X-Gal (X-gal prepared in dimethyl formamide). Plates were incubated overnight at 37°C, and positive (white colonies) were selected for preparing liquid cultures to isolate the recombinant plasmids.

2.11.3 Isolation of Recombinant Plasmids

2.11.3.1 Mini-preparation (Sigma)

The Promega Wizard Prep Kit was used to isolate plasmids from *E.coli* liquid cultures. In two steps, the 2 mL of liquid cultures were transferred into a 1.5 ml microcentrifuge tube and spun at full speed for 2 min in a microcentrifuge to pellet the cells. The liquid medium was removed. To the resulting pellet, 200 µl of cell resuspension solution was added. To the resuspended pellet, 200 µl of cell lysis solution was added, and the tube was inverted four times to mix. 200 µl of neutralizing solution was added and again the tube was inverted 4 times to

mix. The mixture was spun at full speed in a microcentrifuge for 5 min to form a white pellet and clear lysate. A micro-column (supplied with kit) was attached to a sterile 3 ml syringe. 1 ml of column resin solution (supplied with kit) was added followed by the addition of all the cleared lysate. The mixture was slowly passed through the column and discarded. To the syringe (with column still attached) 2 ml of column wash solution was added. The mixture was slowly passed through the column. The column was placed in a sterile 1.5 ml microcentrifuge tube and 50 μ l of sterile water (TE can also be used) was added. After one min incubation at room temperature, the column in the tube was spun at full speed in a microcentrifuge for 20 sec to elute the DNA. 1.5% agarose gel electrophoresis was used to confirm the presence of vector and plasmid. Remaining samples were stored at -20°C for further use.

2.11.3.2 Small Scale Plasmid Isolation

E.coli was grown at 37°C with shaking in 2 ml LB medium with appropriate antibiotic overnight. 1.5 ml was removed to the labeled microfuge tube and centrifuged for 10 sec at full speed at room temperature. The supernatant was removed but for 50-100 μ l. The pellet was resuspended in the remaining supernatant. 300 μ l of TENS (10 mM Tris, 0.1 mM EDTA, 0.1 N NaOH, 0.5% SDS) was added, and the solution was vortexed until mixture had a sticky consistency. The mixture was vortexed for 2-5 sec after being added 150 μ l of 3.0 M NaOAc. The mixture was centrifuged at 1200 g at 4°C for 2 min. The supernatant was transferred to a new tube and was centrifuged for 10 min at 4

°C. The solution was centrifuged for 2 min at 4 °C after the pellet was rinsed with 70% ethanol in TE. The tube was dried by inversion on a Kleenex over heating block. The plasmid DNA was resuspended in 30-50 µl TE and stored it in 4°C fridge.

2.12 Recombinant Plasmid Digested with *Bam*HI and *Kpn*I

Because *Bam*HI and *Kpn*I do not share the same reaction buffer, the digestions were performed separately; digesting with *Bam*HI first, precipitating the DNA, and then digesting with *Kpn*I.

The digestion reactions were set up as follows: 25 µl of DNA (from Wizard Prep isolation), 18.5 µl of sterile water, 5 µl of 10x specific reaction buffer, 0.5 µl of 100x BSA, 1 µl of restriction enzyme (total reaction volume of 50 µl). The mixture was incubated for one hour at 37°C.

The *Kpn*I digestion was set up as the same as above. After digestion 1% agarose gel electrophoresis was used to obtain the inserts. The inserts were cut and gene-cleaned using the GeneClean Kit, and stored at –20°C until needed for further ligations.

2.13 Gene Clean

The gel slice was transferred to pre-weighted microfuge tube to determine its weight. The 2.5-3 volumes of 6 N NaI (FW 149.9, in water) solution was added and incubated at 50 °C with occasional tapping until the agarose slice is completely dissolved (usually 3 to 5 min). Five µl of Glassmilk suspension was

added, mixed by tapping and left on ice for 5 min. The mixture was spun for 5 sec to pellet the Glassmilk. To the pellet, 400 µl of New Wash (50 mM NaCl, 10 mM Tris-Cl, pH 7.5, 2.5 mM EDTA, 50 % ethanol), and resuspended by pipetting up and down. The supernatant was discarded after it was spun. The 2 washes were performed. The tube was drained well by drawing off any remaining wash solution. The Glassmilk pellet was resuspended in 10 µl of dH₂O and incubated at 50°C for 5 min to release the bound DNA. After being centrifuged for 30 sec, the DNA in water was carefully transferred to a clean tube and kept at 4°C.

2.14 DNA Precipitation

DNA was precipitated as follows: 0.1 volumes of 3M ammonium acetate and 2.5 volumes of 100% cold ethanol were added to the digestion mixture (on ice), stored at -20°C overnight, spun at full speed for 15 min (at 4°C) in a microcentrifuge, supernatant was discarded, 200 µl of 70% ethanol were added, sample spun at full speed for 3 min (at 4°C) in a microcentrifuge, the supernatant was removed and the pellet was dried. The dry pellet was resuspended in 25 µl to 50 µl of sterile water.

2.15 pSOL2 vector

The vector pSOL2 (3.8 kb), which was derived from pBI221, was obtained from Dr. Barry Flinn (Canadian Potato Genome Project), containing *Bam*HI restriction site near the Cauliflower mosaic virus 35S promoter, and a *Kpn*I restriction site near the nos terminator. The map of pSOL2 vector is

attached as follow (Figure 2.2). The insertion DNA digested from 2.12 was subcloned into pSOL2 vector. The purpose of this sub-cloning was to add the Cauliflower mosaic virus 35S promoter region to my insertion DNA.

Figure 2. 2 The map of pSOL2 vector.
(Adopted from Dr. Barry Flinn).

2.16 The Plant Transformation Vector--- pSOL6

DH5 α *E.coli* that contained pSOL6 plasmid (10 kb) were also provided by Dr. Barry Flinn (Canadian Potato Genome Project). The map of pSOL6 vector is attached as follow (Figure 2.3). This vector contains *NotI* restriction site in which 35S promoter plus the insertional DNA was inserted. The dephosphorylation of cut pSOL6 had been carried out before doing the ligation. The recombinant pSOL6 plasmid was used to transform *Agrobacterium* after it was isolated from transformed *E. coli*.

Figure 2. 3 The map of the plant transformation vector - pSOL6.
(Adopted from Dr. Barry Flinn).

2.17 Transformation of Recombinant pSOL6 Plasmid Into *Agrobacterium*

Freeze/thaw shock transformation was adopted in my project. The procedures are described as follows: A single colony of LBA4404 *Agrobacterium* was picked and inoculated in 3 ml of LB with selection of Rifampicin (in methanol) at final concentration of 150 µg/ml in a 15 ml Falcon tube. The cells were grown at 30 °C overnight on a roller drum. The 50 ml of LB in a 250 ml flask was inoculated with 0.5 ml of the overnight culture and grown at 30 °C until mid-log (OD₆₀₀ was between 0.5 and 1). Then, the culture was chilled for 5-10 min on ice, centrifuged at 3000 rpm for 5 min at 4°C in the chilled sterile centrifuge tubes. The supernatant was discarded and the remaining liquid was drained by inversion for 30-60 sec. The pellet was resuspended in 1 ml of ice cold 20 mM CaCl₂. 0.1 ml of bacterial suspension was dispensed into each of prechilled 1.5 ml microfuge tube on ice for transformation.

For transformation, 1 µg plasmid DNA was added to one tube that contained 0.1 ml of bacterial suspension, and mixed by tapping with index finger. The mixture was frozen in liquid nitrogen. Then it was thawed for 5 min at 37 °C. To this mixture, 1 ml of LB was added, and transferred to a 15ml Falcon tube, incubated for 2 hrs on a roller drum at 30 °C. The mixture was poured into 1.5 ml microfuge tube and the tube was spun for 5 min to pellet cells. The supernatant was removed and the pellet was resuspended in 100 µl LB. All of the suspension was plated on kanamycin (50 mg/L) and incubated for 2-3 D at 30 °C. Transformed colonies could be visible on the fourth day of incubation.

2.18 Sequencing Recombinant Vector

In order to confirm that the target sequence had been cloned into pGEM-T® Easy Vector, an automated sequencing facility (Canadian Molecular Research Services Inc. 600 Peter Morand Cr. Suite 130 Ottawa, Ont. K1G 5Z3) was used. Both sense and antisense constructs were sent out for sequencing.

2.19 Transformation of *Arabidopsis thaliana*

Floral dip transformation method (Clough *et al.*, 1998) was used for transformation of *Arabidopsis*. *Agrobacterium tumefaciens* strain LBA4404 carrying binary plasmid pSOL6 that contained antisense and sense *CLV1* gene fragment was used to transform *Arabidopsis*. Bacteria were grown to stationary phase in the LB liquid culture with kanamycin and rifampicin. Cultures were typically started from a 1: 100 dilution of a smaller overnight culture and grown for roughly 18-24 hr. Cells were harvested by centrifugation for 20 min at room temperature at 5500 g and then resuspended in the LB to a final OD₆₀₀ of approximately 0.80 prior to use. The inoculation medium contained 5.0% sucrose and 0.05% (i.e. 500 µl per liter) Silwet L-77 (Osi Specialties, Inc., Danbury, CT, USA). For floral dip, the inoculum was added to a beaker.

The primary bolts were clipped. When the secondary bolts were about 2-10 cm (few open flowers), plants were inverted into this suspension such that all aboveground tissues were submerged, and plants were then removed after 3-5

sec of gentle agitation. Dipped plants were removed from beaker, placed in a plastic tray and covered with a tall clear plastic dome to maintain humidity. Plants were left in a low light or dark location overnight and returned to greenhouse the next day. Care was taken to keep domed plants out of direct sunlight. Domes were removed approximately 12-24 hr after treatment.

Plants were grown for a further 3-5 weeks until siliques were brown and dry, keeping the bolts from each pot together and separated from neighboring pots using plastic cone. Seeds were harvested by gentle pulling of grouped inflorescences through finger over a piece of clean paper. The majority of the stem and pod material was removed from paper by using a fine screener. Seeds were stored in microfuge tubes and kept at 4°C fridge.

2.20 Selection of Putative Transformants Using an Antibiotic Marker

Seeds were surface sterilized as described in 2.1. To select for transformed plants, sterilized seeds were suspended in 0.1% sterile agarose and plated on kanamycin selection plate at density of approximately 3000 seeds per 150 X 15 mm 2 plate, cold-treated for 2 D in 4°C fridge, and then grown for 7-10 D in the long-day condition growth chamber. Selection plates contained ½ X MS medium (Sigma Chemicals # M-5519), 0.8 % agar (Sigma Chemicals # A-1296), 50µg /ml kanamycin monosulfate. Petri plates and lids were sealed with parafilm. Transformants were identified as kanamycin-resistant seedlings that produced green true leaves and well-established roots within the selective medium. Some

transformants were grown to maturity by transplanting, preferably after the development of 3-5 adult leaves, into heavily moistened potting soil.

2.21 Genomic DNA Isolation from *Arabidopsis*

Genomic DNA was isolated from whole *Arabidopsis thaliana* plants (Columbia strain) using the QIAGEN DNeasy Plant Mini Kit. Plant tissue was ground under liquid nitrogen to a fine powder using a mortar and pestle. Approximately 100 mg of ground tissue was transferred to a 1.5 mL microcentrifuge tube, with liquid nitrogen, and 400 µl of Buffer AP1 was immediately added. The mixture was vigorously vortexed. The mixture was incubated for 10 min at 65 °C, and mixed by inverting for 2-3 times during incubation. In order to precipitate detergent, proteins, and polysaccharides, 130 µl of Buffer AP2 was added to the lysate and incubated for 5 min on ice. After incubation, the lysate was centrifuged for 5 min at full speed. Then the lysate was applied to the QIAshredder spin column (lilac) sitting in a 2 ml collection tube and centrifuged for 2 min at maximum speed. The flow-through fraction was transferred to a new tube without disturbing the cell-debris pellet. To the cleared lysate, 675 µl of Buffer AP3/E was added. The 650 µl of the mixture was applied to the DNeasy mini spin column sitting in a 2 ml collection tube, and centrifuged for 1 min at 8000 rpm, and the flow-through was discarded. To the DNeasy column, 500 µl of Buffer AW (wash buffer) was added, centrifuged for 1 min at 8000 rpm, and the flow-through was discarded. Another 500 Buffer AW was added to DNeasy column and centrifuged for 2 min at maximum speed to dry the

membrane. The DNeasy column was transferred to a 1.5 ml microcentrifuge tube, and 100 μ l of preheated (65 °C) Buffer AE was pipetted directly onto the DNeasy membrane. Incubated it for 5 min at room temperature and centrifuged for 1 min at 8000 rpm to elute. The second elution was repeated. Agarose (1%) gel electrophoresis was used to confirm the presence of DNA and quantify concentration of DNA samples. The remaining sample was stored at -20°C

2.22 Southern Blot

The 10 μ l genomic DNA of 13 different transgenic *Arabidopsis* lines was digested by *Bam*HI and *Hind*III respectively for 2 hrs at 37 °C. The digested samples were electrophoresed on the 1% agarose gel. After the gel was rinsed in dH₂O for 5 min, the gel was soaked in 0.25 M HCl at RT for 30 min with gentle rocking to shear longer DNA into small one. The gel was transferred to 0.4M NaOH with gentle shaking for 20 min after it was briefly rinsed in the dH₂O. The purpose of this step was to denature and separate DNA into single strands.

A support for transfer was constructed by placing a 1 cm high Plexiglas block that was wrapped in 3 MM filter paper into a 3L glass-baking dish. Enough 0.4 NaCl was added to dish to just reach the top of support. A piece of nylon membrane (Amersham), which was slightly larger than the gel (9 cm X 11 cm), was set on the top of the gel. 2 pieces of 3 MM filter paper, the same size as the gel, were stacked on the top of membrane followed by a 10-15 cm high stack of paper towels that were slightly smaller than the gel. The capillary transfer through the gel and up into the paper towels were allowed to proceed for 12 hrs. After

transfer, the membrane was briefly soaked in 6X SCC to remove residual agarose. After the membrane was cross-linked under UV for 5 min, it was dried in an 80 °C oven for 2 hrs. Then transfer efficiency was checked using UV light before being wrapped in foil for storage at room temperature.

A “ ready to go “ DNA labeling (minus dCTP) kit (Pharmacia) was used following the manufacture’s instructions. About 25 ng linear DNA (probe) in 45 µl TE buffer (pH 8) was denatured in the boiling water for 5 min, and quickly chilled on ice for another 5 min. the denatured DNA was added to the tube containing the “Ready to go” reaction bead followed by adding 5 µl of α -³²P labeled dCTP. The reaction was gently mixed 12 times by pipetting and incubated at 37 °C for 30 min. The reaction was stopped by adding 2 µl of 0.5 M EDTA.

Spun column chromatography was used to remove unincorporated nucleotides. The bottom end of 1 ml syringe was plugged with glass wool and it was filled with Sephadex G –50 beads previously equilibrated in 1X STE (100 mM NaCl , 20 mM Tris-Cl pH 7.5, 10 mM EDTA). The Sephadex G-50 matrix was packed down to a volume of 0.9 ml by repeated spins at 3, 000 rpm in a swinging-bucket centrifuge. The radio labeling reaction was loaded onto the top of spun column. The column was centrifuged at 3, 000 rpm for 4 min forcing the radiolabeled probe to be eluted from the column into a centrifuge tube. The probe was denatured in the boiling water for 5 min before use.

The transferred membrane was put into a glass screw –cap hybridization tube. The pre-hybridization (1% BSA, 1mM EDTA, 0.5 M Na-PO₄, 7% SDS) buffer was added at 0.05 ml per cm² of membrane. The tube was loaded into

rotating Hybridization Incubator Model 310 (Robbins Scientific) and incubated at 65 °C for 3 hrs.

The DNA probe was mixed with salmon sperm DNA (20 µg per ml hybridization solution) and it was denatured at boiling water for 5 min. the mixture was quick-chilled and then added to hybridization tube.

Hybridization was performed at 65 °C for 12 hrs. Stringency washes were performed at the conclusion of incubation. The hybridization solution was discarded; 50 ml of 2X SSC were added to hybridization tube and incubated at 68 °C for 15 min.

The membrane was allowed to air-dry slightly prior exposure to Kodak auto-radiographic film. The membrane and film were stored at –20 °C in a light-tight cassette until the desired signal was reached.

After sufficient exposure of film to the labeled membrane, the film was developed for 1 min in GBX developer and replenisher (Kodak). The film was rinsed in water for 1-2 min, soaked in GBX fixer and replenisher (Kodak) and then rinsed for 5 min in water before it was left to dry.

2.23 Northern Blot

In order to prepare the RNA samples for the Northern Blot, the samples first had to be electrophoresed under denaturing conditions on an agarose-formaldehyde gel. The agarose-formaldehyde gel was created by first dissolving 1.0 g of agarose in 72 ml of DEPC treated water and allowed it to cool to 60 °C. To this, 10 ml of 10x MOPS (0.4 M MOPS pH 7.0, 0.1 M sodium acetate, 0.01 M

EDTA) and 18 ml of 12.3 M formaldehyde was added. Once the gel was poured, approximately 450 ml of 1x MOPS was added to cover the gel to a depth of 1 mm. Then, the gel was electrophoresed for 15 min at 95 V to remove any possible RNase contamination. The RNA samples were prepared by adding 0.454 μ l of 10x MOPS, 0.81 μ l of 12.3 M formaldehyde, and 2.27 μ l of formamide to 1 μ l of RNA. The contents were then vortexed, briefly centrifuged, and incubated for 15 min at 55 °C. Then, 10 μ l of formaldehyde loading buffer was added to each sample before being loaded in the wells of the gel. The formaldehyde loading buffer was created from a mixture of 720 μ l of formamide, 160 μ l of 10x MOPS, 260 μ l of 37% formaldehyde, 100 μ l of DEPC water, 100 μ l of 10 ng/ml ethidium bromide, 80 μ l of sterile glycerol, and 80 μ l of saturated Orange G in sterile distilled water. The gel was then photographed using the Alpha Imager IS-2000.

The Northern blot was then performed using a slightly modified version of the method outlined in Brown & Mackey (1997). To begin, the agarose-formaldehyde gel was placed in 10 gel volumes of 20x SSC (3 M NaCl and 0.3 M Na₃citrate · 2H₂O) for 45 min. To transfer the RNA from the gel to the membrane, a treated RNase free dish was first placed upside down in a larger RNase free Pyrex tray. Then, a small piece of 3 mm Whatman paper was placed on top of the smaller tray, with a larger piece allowed to hang down into the larger Pyrex tray. The largest Pyrex tray was then filled with enough 20x SSC to cover most of the longer 3 mm Whatman paper. The gel was then placed with the wells facing down on the Whatman paper. A glass pipette was used to roll

out any air bubbles between the gel and the Whatman paper. A piece of nylon membrane was then cut to the exact dimensions of the gel and soaked in distilled water for 10 min before being placed on the back of the gel. Again, a glass pipette was used to roll out any air bubbles. The surface of the membrane was then soaked with 20x SSC. Five more pieces of 3 mm Whatman paper, cut to size, were then placed on top of the membrane. Finally, a stack of paper towels were cut to size and placed on the very top with a 1 L bottle to act as weight. The apparatus was then left overnight to allow for the RNA transfer to occur. Once constructed, the apparatus was left overnight.

In the next morning, the apparatus was disassembled and the wells were marked onto the membrane with a pencil. The membrane was then rinsed in 2x SSC and placed on 3mm Whatman paper to dry (Brown & Mackey, 1997). To covalently attach the RNA to the membrane, UV irradiation was performed by placing the membrane face down on the UV box for 5 min. The membrane was then placed between 2 sheets of 3mm Whatman paper and placed on the vacuum drier at 80 °C for 1.5 hours. Finally, the membrane was wrapped in plastic wrap and tin foil, sealed in an RNase-free plastic bag, and placed at 4 °C for storage.

The hybridization analysis was performed using radioactively labeled *CLV1* probe. A prehybridization step was performed by placing the rolled membrane, with the RNA side on the inside, in a hybridization tube with 20 ml of ExpressHyb Hybridization Solution (or CHURCH buffer). The hybridization tube was then placed in the oven at 65 °C for 1.5 hours. During this time, the probes

were prepared by first diluting 2 µl of CLV1 probe in 43 µl of TE buffer (pH 8.0). Then, the probes were denatured at 100 °C for 5 min in a water bath before being placed on ice for 5 min to cool. After being centrifuged briefly, the probes were transferred to a reaction tube from the Rediprime II DNA Labeling System (For more information on Rediprime II DNA Labeling System see www5.amershambiosciences.com.)

by Amersham Biosciences. The reaction tubes did not contain dCTP, which allows for the incorporation of radioactive dCTP. For this reason, 5 µl of Rediprime [³²P] dCTP was added to the reaction tube prior to mixing by pipette. Then, the reaction tube was incubated at 37 °C for 30 min. The reaction was stopped using 5 µl of 0.2 M EDTA prior to being placed in a 100 °C water bath for 5 min. Finally the reaction tube was placed on ice for 5 min prior to being added to the hybridization tubes. These tubes were then returned to the oven where they remained at 65 °C overnight. The hybridization buffer was poured off, and equal volume of 0.2X SSC/0.1 % SDS was added, and incubated for 15 min at room temperature. Performed autoradiography.

2.24 Preparation of the Hand Section of Hypocotyls and Stems

The piece of hypocotyls (or stems) of Arabidopsis was held between the thumb and index finger, so that the cutting surface was perpendicular to axis of the axis of hypocotyls. Several thin sections were cut by drawing the blade toward me over the hypocotyl. The sections were removed from blade by gently spraying them off with a wash bottle into one of the small dishes of water. The

thinnest ones were chosen to be the best ones. Two or three drops of Toluidine Blue pH 4.4 were placed into a small glass dish and the best sections were transferred into the dish using the flat ended applicator stick, the left for 30 sec to 1 min. The stained sections were transferred to a dish of clean water in order to rinse off excess stain. The sections were mounted onto a drop of water on glass slide, and the sections were covered with glass cover-slip for microscopic cellular examination.

3. Results

3.1 Cloning of *CLV1* into a Plant Transformation Vector

3.1.1 RNA Isolation from *Arabidopsis*

Total RNA was isolated from whole plants of *Arabidopsis*. From agarose gel separation, it was estimated that there was 0.8µg/µl of *Arabidopsis* RNA. Based on the banding patterns of the 25S (4.7 kb) and the 18S (1.9 kb) ribosomal RNA subunit as shown in the Figure 3.1A, the RNA was determined to be of good quality.

3.1.2 RT-PCR Results

cDNA was made from *Arabidopsis* RNA as being a DNA template, and the fragment (605 bp, see Figure 3.2) of *CLV1* was isolated by running the RT-PCR. The observed band was sharp and distinct (Figure 3.1B); indicating only one size of product was amplified.

3.1.3 Ligation of PCR products into pGEM®-T Easy Vector

Both sense and antisense *CLV1* cDNA fragments were ligated into the pGEM®-T Easy vector that has TA overhang to accept PCR products, and *Bam*HI, *Kpn*l restriction enzyme sites were introduced to the insertional DNA by the pre-designed primers.

Ligation turned out to be successful, because the white colonies were observed from positive transformed *E. coli* colonies. The untransformed colonies showed blue color, because the *lac Z* gene was not disrupted by the insert of interest. The recombinant pGEM-T plasmids were isolated by using Mini-Prep kit,

and gel picture showed as Figure 3.3A). These plasmids were named pSYH1 (sense construct) and pSYH2 (antisense construct).

3.1.4 Restriction Digestion to Confirm the Presence of an Insert

Following plasmid isolation, the presence of an insert in the vector was confirmed by digestion with *Not* I. It was confirmed that there were no *Not* I digest sites within the known *Arabidopsis CLV1* sequence to ensure one insert to be released. The cut and uncut samples were simultaneously electrophoresed in 1% agarose gel (Figure 3.3B). The digest product (Lane 3, 5, 7 and 9) appeared as a sharp band, indicating complete release of the insert (627 bp), and the size of linearized pGEM-T easy vector was exactly 3015 bp. The uncut plasmids just showed two bands, namely supercoiled and open circular.

3.1.5 Insert Sequence Confirmation

The plasmids (pSYH1 and pSYH2) with confirmed insert were sent away to Canadian Molecular Research Services Inc. for sequencing. Results showed that proper inserts with proper orientation were isolated, and cloned from *Arabidopsis*.

3.1.6 Fragment Transfer from pGEM[®]-T Easy Vector to pSOL2

The amplified products were then cloned into pSOL2 to add the 35S promoter region to the *CLV1* sense and antisense fragments. The pSOL2 plasmid contains ampicillin resistance site and it was isolated from *E. coli*, and then cut by *Bam*HI and *Kpn* I. The linearized pSOL2 was ligated with the inserts from pSYH1 and pSYH2 respectively, and transformed into *E. coli*. The agarose gel in Figure 3.4A shows a pSOL2 with an insert in lane 4. Recombinant

plasmids were named pSYH3 (sense construct) and pSYH4 (antisense construct).

3.1.7 Restriction Digest of pSYH3 and pSYH4 to Release the Gene with the 35S Promoter Region.

The pSYH3 and pSYH4 plasmids were digested by *Not*I after they were isolated from *E. coli*. The *Not*I sites are located outside the CaMV 35 S Promoter region and Nos-ter region, so the released insert contained the CaMV 35S Promoter region, *CLV1* fragments in either the sense or antisense orientation and Nos-ter region (Figure 3.4B). The inserts are now ready for subcloning to the expression vector pSOL6.

3.18 Fragment Transfer from pSOL2 to pSOL6

The inserts cut from pSYH3 and pSYH4 were ligated with the pSOL6, which was cut by the same restriction enzyme *Not*I,. The presence of Kan^R colonies did not necessarily mean the plasmid contained the insert because empty pSOL6 vector (the pSOL6 without insert) could have kanamycin resistance. So, primers were designed based on the 35S promoter region and nos-ter region, and colony-PCR was engaged to confirm the presence of the insert (Figure 3.4C). The PCR products of sense and antisense plasmids were sent to be sequenced, and the results matched the expected sequence. Recombinant plasmids were designated pSYH5 (sense construct) and pSYH6 (antisense construct). These plasmids were then transformed into *Agrobacterium* for subsequent *Arabidopsis* flower-dip transformation.

Figure 3. 1 Agarose gel picture showing total RNA (A) and RT-PCR products. Samples were run on a 1% agarose gel containing 5 µl of 10 mg/ml EtBr at 83 volts.

A) RNA isolation from *Arabidopsis Colombia* whole plant.

Lane1: Five µl isolated *Arabidopsis Colombia* RNA sample using QIAEN RNeasy kit.

Lane2: Five µl 1 kb DNA ladder (0.42 µg) from Life Technologies.

B) *Arabidopsis Colombia* RT-PCR results.

Lane1: Five µl 1 kb DNA ladder.

Lane 2&3. Five µl of amplified *Arabidopsis* 605 bp DNA from RT-PCR.

A

B

Figure 3. 2 The part of double strand sequence of *CLV1* in *Arabidopsis*.
The length of whole *CLV1* cDNA sequence is 3388 bp. Both forward and reverse primers are indicated as bolded and underlined. The length of amplified DNA is 605 bp.

Forward Primer

481 TCCGGATGCT CACTGTTCTT TCTCCGGCGT CTCATGTGAC GACGATGCTC GTGTTATCTC
AGGCCTACGA GTGACAAGAA AGAGGCCGCA GAGTACACTG CTGCTACGAG CACAATAGAG

541 TCTCAACGTC TCCTTCACTC CTTTGTTTGG TACAATCTCA CCAGAGATTG GGATGTTGAC
AGAGTTGCAG AGGAAGTGAG GAAACAAACC ATGTTAGAGT GGTCTCTAAC CCTACAACTG

601 TCATTTGGTG AATCTAACTT TAGCTGCCAA CAACTTCACC GGTGAATTAC CATTGGAGAT
AGTAAACCAC TTAGATTGAA ATCGACGGTT GTTGAAGTGG CCACTTAATG GTAACCTCTA

661 GAAGAGTCTA ACTTCTCTCA AGGTTTTGAA TATCTCCAAC AATGGTAACC TTACTGGAAC
CTTCTCAGAT TGAAGAGAGT TCCAAAACCTT ATAGAGGTTG TTACCATTGG AATGACCTTG

721 ATTCCCTGGA GAGATTTTAA AAGCTATGGT TGATCTTGAA GTTCTTGACA CTTATAACAA
TAAGGGACCT CTCTAAAATT TTCGATACCA ACTAGAACTT CAAGAACTGT GAATATTGTT

781 CAATTTCAAC GGTAAGTTAC CACCGGAGAT GTCAGAGCTT AAGAAGCTTA AATACCTCTC
GTTAAAGTTG CCATTCAATG GTGGCCTCTA CAGTCTCGAA TTCTTCGAAT TTATGGAGAG

841 TTTCGGTGGA AATTTCTTCA GCGGAGAGAT TCCAGAGAGT TATGGAGATA TTCAAAGCTT
AAAGCCACCT TTAAAGAAGT CGCCTCTCTA AGGTCTCTCA ATACCTCTAT AAGTTTCGAA

901 AGAGTATCTT GGTCTCAACG GAGCTGGACT CTCCGGTAAA TCTCCGGCGT TTCTTTCCCG
TCTCATAGAA CCAGAGTTGC CTCGACCTGA GAGGCCATTT AGAGGCCGCA AAGAAAGGGC

961 CCTCAAGAAC TTAAGAGAAA TGTATATTGG CTA CTACTACAAC AGCTACACCG GTGGTGTTC
GGAGTTCTTG AATTCTCTTT ACATATAACC GATGATGTTG TCGATGTGGC CACCACAAGG

1021 ACCGGAGTTC GGTGGTTTAA CAAAGCTTGA GATCCTCGAC ATGGCGAGCT GTACTCTCAC
TGGCCTCAAG CCACCAAATT GTTTCGAACT CTAGGAGCTG TACCGCTCGA CATGTGAGTG

1081 CGGAGAGATT CCGACGAGTT TAAGTAACCT GAAACATCTA CATACTCTGT TTCTTCACAT
GCCTCTCTAA GGCTGCTCAA ATTCATTGGA CTTTGTAGAT GTATGAGACA AAGAAGTGTA

Reverse Primer

Figure 3. 3 Recombinant pGEM-T vector and digestion.

A) Recombinant pGEM-T vector that contained the insertional DNA.

Lane1: Five μ l (0.42 μ g) 1 kb DNA ladder.

Lane 2-4. Five μ l recombinant pGEM[®]-T Easy plasmid (length: 3015 bp + 605 bp = 3,620 bp) that contained *CLV1* fragment with sense orientation.

Lane 5 showed a pGEM-T vector without insertional DNA as a control.

Lane 6-9. Five μ l recombinant pGEM[®]-T Easy plasmid that contained *CLV1* fragment with antisense orientation

B) pSYH1(sense construct) and pSYH2 (antisense construct) digested with *Not* I.

Lane1: Five μ l (0.42 μ g) 1 kb DNA ladder.

Lane 2, 4, 6 and 8: Five μ l uncut recombinant pGEM[®]-T Easy plasmid.

Lane 3, 5, 7, and 9: Five μ l cut recombinant pGEM[®]-T Easy plasmid.

A

B

Figure 3. 4 The recombinant pSOL2 and pSOL6 plasmids.

A) The recombinant pSOL2 plasmid.

Lane1: Five μ l (0.42 μ g) 1 kb DNA ladder.

Lane 2, 3: Five μ l pSOL2 vector only.

Lane 4: Five μ l pSOL2 plasmid with insert.

B) The recombinant pSOL2 plasmids cut by *Not* I.

Lane1: five μ l (0.42 μ g) 1 kb DNA ladder.

Lane 2, 3: five μ l pSYH3 plasmid (sense orientation).

Lane 4, 5: five μ l pSYH4 plasmid (antisense orientation).

C) Colony-PCR of pSYH5 and pSYH6 plasmids.

Lane1: five μ l (0.42 μ g) 1 kb DNA ladder.

Lane 2, 3: five μ l PCR product of SYH5 plasmid (sense orientation).

Lane 4, 5: five μ l PCR product of pSYH6 plasmid (antisense orientation).

A

B

C

3.2 *Arabidopsis* Transformation

3.2.1 Flower-dip Transformation

Arabidopsis were transformed as previously described (Clough *et al.*, 1998) and the treated plants were grown at 20-22 °C in the long day conditions to maturity (another 20 days). The seed were then harvested and screened for transformants on a MS solid medium with kanamycin selection.

3.2.2 Result of Transformation

Seeds were planted on the cheesecloth, which was placed on the solid MS media with Kanamycin⁵⁰ after the seeds had been sterilized and stratified at 4 °C for 4 D. The sealed Petri dishes were incubated in the long-day condition growth chamber for 10 days. Kan^R seedlings had green cotyledons and true leaves with roots protruding into the media. Kan^S seedlings were white and usually did not have any roots in the solid media. They became yellowish and eventually died; seeds that failed to germinate were ignored (Figure 3.5A, B). Out of the 25,000 seeds that were tested from the treated plants, 41 antisense transformed lines, and 7 sense-transformed lines were stably transformed. Four plants were male sterile in the primary transformation in the antisense transformed lines. These plants were designated T₁. Seven-day-old plantlets were numbered and transferred into little pots containing multi-purpose compost to grow to maturity and harvested individually.

Figure 3. 5 The appearance of the transformed seedlings.

- A) The picture on the left shows seeds screened on Kan MS solid media.
- B) The picture on the right shows that the transgenic plant has true leaves while WT plant did not.
- C) Seeds of Anti-L31 in T₃ generation were screened on Kan Petri dish. Both homozygous and WT plants were segregated out.

A

B

C

3.3 Genetic Analysis of Transgenic Plants

3.3.1 Phenotypic Analysis of Transgenic Lines

The 41 antisense transformed lines, and 7 sense transformed were grown individually. The flowers of these plants were examined to see if the homozygous transgenic plants could mimic the phenotype of *clv1* mutant that contains a greater number of floral organs. Totally, 8 antisense lines, which accounted for 19.51% of total antisense transformed plants, mimicked the phenotype of *clv1* mutant. This suggests that the antisensed region, which lies in the LRR region of *CLV1* gene (Figure 3.6), results in a phenotype similar to the strongest mutant alleles *clv1-4* and *clv1-8*, which contained missense mutations in the LRR domain (Clark *et al.*, 1997).

The phenotype of T₃ transgenic plants varied. Typically, Anti-L8, 9, 16, 18, 23, 27, 30, and L32 had extra petals, thus mimicking the *clv1* mutant phenotype. Two interesting lines were Anti-L22 that had 3 petals, and Anti-L38 that had deformed petals, and the angle between the petals was changed, while the angle between the petals of WT plant always keeps 90° (Figure 3.7A-I).

Further more, the floral meristem of Anti_L41 was chosen to compare with that of WT, and it was found to be enlarged compared to that of WT (Figure 3.7J). This is consistent with the *clv* floral meristem that can be twice as tall as the WT floral meristem at the earliest stage of organ initiation (Clark *et al.*, 1997). Analysis of maturing flowers revealed that *clv* floral meristem continued to proliferate and gave rise to a large mass of undifferentiated cells in the center of the flower, instead of terminating like the WT floral meristem.

It should be pointed out that some transgenic plants did not mimic the *clv1* mutant phenotype in the T₂ generation but showed the typical *clv1* mutant phenotype in later generations. For instance, Anti-L5 mimicked *clv1* phenotype in T₄ generation that was grown in the short day condition growth chamber.

Figure 3. 6The *CLV1* gene and protein sequence.

The DNA sequence corresponding to the *CLV1* transcript is shown with the predicted amino acid sequence shown below. The nucleotide sequence and their corresponding protein sequence are numbered at the left. The paired cysteines flanking the LRR region are underlined. The predicted transmembrane sequence has a double strike through it. In the kinase domain, the conserved regions are highlighted and indicated with Roman numerals that are adopted from Clark *et al* (1997).

301 CTGAGAGGACACTAAAAAATGGCGATGAGACTTTTGAAGACTCATCTTCTGTTTCTGCA
 1 M A M R L L K T H L L F L H
 361 TCTGTATCTATTTTTCTCACCATGTTTCGCTTACACTGACATGGAAGTTCTTCTCAATCT
 15 L Y L F F S P C F A Y T D M E V L L N L
 421 CAAATCCTCCATGATTGGTCCTAAAGGACACGGTCTCCACGACTGGATTCACTCATCTTC
 35 K S S M I G P K G H G L H D W I H S S S
 481 TCCGGATGCTCACTGTTCTTTCTCCGGCGTCTCATGTGACGACGATGCTCGTGTTATCTC
 55 P D A H C S F S G V S C D D D A R V I S
 541 TCTCAACGTCTCCTTCACTCCTTTGTTTGGTACAATCTCACCAGAGATTGGGATGTTGAC
 75 L N V S F T P L F G T I S P E I G M L T
 601 TCATTGGTGAATCTAACTTTAGCTGCCAACAACTTCACCGGTGAATTACCATTGGAGAT
 95 H L V N L T L A A N N F T G E L P L E M
 661 GAAGAGTCTAACTTCTCTCAAGGTTTGAATATCTCCAACAATGGTAACCTTACTGGAAC
 115 K S L T S L K V L N I S N N G N L T G T
 721 ATTCCCTGGAGAGATTTTAAAGCTATGGTTGATCTTGAAGTTCTTGACACTTATAACAA
 135 F P G E I L K A M V D L E V L D T Y N N
 781 CAATTTCAACGGTAAGTTACCACCGGAGATGTCAGAGCTTAAGAAGCTTAAATACCTCTC
 155 N F N G K L P P E M S E L K K L K Y L S
 841 TTTCCGTGGAAATTTCTTCAGCGGAGAGATTCCAGAGAGTTATGGAGATATTCAAAGCTT
 175 F G G N F F S G E I P E S Y G D I Q S L
 901 AGAGTATCTTGGTCTCAACGGAGCTGGACTCTCCGGTAAATCTCCGGCGTTTCTTTCCCG
 195 E Y L G L N G A G L S G K S P A F L S R
 961 CCTCAAGAACTTAAGAGAAATGTATATTGGCTACTACAACAGCTACACCGGTGGTGTTC
 215 L K N L R E M Y I G Y Y N S Y T G G V P
 1021 ACCGGAGTTCCGGTGGTTTAACAAAGCTTGAGATCCTCGACATGGCGAGCTGTACACTCAC
 235 P E F G G L T K L E I L D M A S C T L T
 1081 CGGAGAGATTCCGACGAGTTTAAGTAACCTGAAACATCTACATACTCTGTTTCTTTCACAT
 255 G E I P T S L S N L K H L H T H I
 1141 CAACAACTTAACCGGTCAATATACCACCGGAGCTTTCCGGTTTAGTCAGCTTGAAATCTCT
 275 N N L T G H I P P E L S G L V S L K S L
 1201 CGATTTATCAATCAATCAGTTAACCGGAGAAATCCCTCAAAGCTTCATCAATCTCGGAAA
 295 D L S I N Q L T G E I P Q S F I N L G N
 1261 CATTACTCTAATCAATCTCTTTCAGAAACAATCTCTACGGACAAATACCAGAGGCCATCGG
 315 I T L I N L F R N N L Y G Q I P E A I G
 1321 AGAATTACCAAACTCGAAGTCTTCGAAGTATGGGAGAACAATTTACGTTACCAATTACC
 335 E L P K L E V F E V W E N N F T L Q L P
 1381 GGCGAATCTTGGCCGGAACGGGAATCTAATAAGCTTGATGTCTCTGATAATCATCTCAC
 355 A N L G R N G N L I K L D V S D N H L T
 1441 CGGACTTATCCCCAAGGACTTATGCGAGAGTGAGAAATTAGAGATGTTAATTCTCTCTAA
 375 G L I P K D L C R G E K L E M L I L S N
 1501 CAACTTCTTCTTTGGTCCAATTCCAGAAGAGCTTGGTAAATGCAAACTCCTTAACCAAAAT
 395 N F F F G P I P E E L G K C K S L T K I
 1561 CAGAATCGTTAAGAATCTTCTCAACGGCACTGTTCCGGCGGGGCTTTTCAATCTACCGTT
 415 R I V K N L L N G T V P A G L F N L P L
 1621 AGTTACGATTATCGAACTCACTGATAATTTCTTCTCCGGTGAACCTCCGGTAACGATGTC
 435 V T I I E L T D N F F S G E L P V T M S
 1681 CGGCGATGTTCTCGATCAGATTTACCTCTCTAACAACCTGGTTTTCCGGCGAGATTCCACC
 455 G D V L D Q I Y L S N N W F S G E I P P
 1741 TGCGATTGGTAATTTCCCCAATCTACAGACTCTATTCTTAGATCGGAACCGATTTCGCGG
 475 A I G N F P N L Q T L F L D R N R F R G
 1801 CAACATTCGAGAGAAAATCTTCGAATTGAAGCATTTATCGAGGATCAACACAAGTGCGAA
 495 N I P R E I F E L K H L S R I N T S A N
 1861 CAACATCACCGGCGGTATTCCAGATTCAATCTCTCGCTGCTCAACTTTAATCTCCGTCGA
 515 N I T G G I P D S I S R C S T L I S V D
 1921 TCTCAGCCGTAACCGAATCAACGGAGAAATCCCTAAAGGGATCAACAACGTGAAAACTT
 535 L S R N R I N G E I P K G I N N V K N L
 1981 AGGAACTCTAAATATCTCCGGTAATCAATTAACCGGTTCAATCCCTACCGGAATCGGAAA
 555 G T L N I S G N Q L T G S I P T G I G N
 2041 CATGACGAGTTTAACAACTCTCGATCTCTTTTCAACGATCTCTCCGGTAGAGTACCACT
 575 M T S L T T L D L S F N D L S G R V P L

2101 CGGTGGTCAATTCTTGGTGTTCACGAAACTTCCTTCGCCGGAACACTTACCTCTGTCT
 595 G G Q F L V F N E T S F A G N T Y L C L
 2161 CCCTCACCGTGTCTCTTGTCCAACACGGCCAGGACAAACCTCCGATCACAATCACACGGC
 615 P H R V S C P T R P G Q T S D H N H T A
 2221 GTTGTCTCACCGTCAAGGATCGTAATCACGGTTATCGCAGCGATCACCGGTTTGATCCT
 635 L F S P S R ~~I V I T V I A A I T G L I L~~
 2281 AATCAGTGTAGCGATTCTGTCAGATGAATAAGAAGAAGAACAGAAATCTCTCGCCTGGAA
 655 ~~I S V A I~~ R Q M N K K K N Q K S L A W K
 2341 ACTAACCGCCTTCCAGAACTAGATTCAAATCTGAAGACGTTCTCGAGTGTCTTAAAGA
 675 L T A F Q K L D F K S E D V L E C L K E
 2401 AGAGAACATAATCGGTAAAGGCGGAGCTGGAATTGTCTACCGTGGATCAATGCCAAACAA
 695 E N I I ~~G K G G A G~~ I V Y R G S M P N N
 I
 2461 CGTAGACGTGCGGATTAAACGACTCGTTGGCCGTGGGACCGGGAGGAGCGATCATGGATT
 715 V D V A I ~~K~~ R L V G R G T G R ~~S D H G F~~
 II III
 2521 CACGGCGGAGATTCAAACCTTTGGGGAGAATCCGCCACCGTCACATAGTGAGACTTCTTGG
 735 ~~T A E I Q T L G R I~~ R H R H I V R L L G
 2581 TTACGTAGCGAACAAGGATACGAATCTCCTTCTTTATGAGTACATGCCTAATGGAAGCCT
 755 Y V A N K D T N L L L Y E Y M P N G S L
 2641 TGGAGAGCTTTTGCATGGATCTAAAGGTGGTCATCTTCAATGGGAGACGAGACATAGAGT
 775 G E L L H G S K G G H L Q W E T R H R V
 2701 AGCCGTGGAAGCTGCAAAGGGCTTGTGTATCTTCACCATGATTGTTACCATTTGATCTT
 795 A V E A A K G L C Y L H H D C S P L I L
 2761 GCATAGAGATGTTAAGTCCAATAACATTCTTTTGGACTCTGATTTTGAAGCCCATGTTGC
 815 ~~H R D V K S N N I L L D S D F E A H V A~~
 vib
 2821 TGATTTTGGGCTTGCTAAGTTCTTAGTTGATGGTGCTGCTTCTGAGTGTATGTCTTCAAT
 835 ~~D F G~~ L A K F L V D G A A S E C M S S I
 VII
 2881 TGCTGGCTCTTATGGATACATCGCCCCAGAGTATGCATATACCTTGAAAGTGGACGAGAA
 855 A ~~G S Y G Y I A P E Y A~~ Y T L K V D E K
 VIII
 2941 GAGTGATGTGTATAGTTTCGGAGTGGTTTGTGGAGTTAATAGCTGGGAAGAAACCTGT
 875 S ~~D V Y S F G V V L L E L I A G K K P V~~
 IX
 3001 TGGTGAATTTGGAGAAGGAGTGGATATAGTTAGGTGGGTGAGGAACACGGAAGAGGAGAT
 895 G E F G E G V D I V R W V R N T E E E I
 3061 AACTCAGCCATCGGATGCTGCTATTGTTGTTGCGATTGTTGACCCGAGGTTGACTGGTTA
 915 T Q P S D A A I V V A I V D P R L T G Y
 3121 CCCGTTGACAAGTGTGATTCTGTGTCAAGATCGCAATGATGTGTGTGGAGGAAGAAGC
 935 P L T S V I H V F K I A M M C V E E E A
 3181 CGCGGCAAGGCCTACGATGAGGGAAGTTGTGCACATGCTCACTAACCTCCTAAATCCGT
 955 A A R P T M R E V V H M L T N P P K S V
 3241 GGCGAACTTGATCGCGTTCTGACCCAAGCAAGATAAATATGAAAATAAGATGCGAAATGT
 975 A N L I A F

Figure 3. 7 Comparison of WT and transgenic antisense flowers, floral meristem and siliques in the T₂ generation.

Note the varied number of petals in the transgenic lines, and the size of floral meristem of transgenic plants was enlarged compared to that of WT plants.

A: WT flower.

B: Anti_L5, 6 petals. *clv1* phenotype showed in T4 generation.

C: Anti_L9, 5 petals.

D: Anti_L16, extra petal.

E: Anti_L22, 3 petals.

F: Anti_L23, 5 petals.

G: Anti_L30, 5 petals.

H: Anti_L32, 5 petals.

I: Anti_L38, deformed petals.

J: Anti_L41, enlarged floral meristem.

K: The comparison of shape and size of silique between mutant and WT in Landsburg and Columbia ecotype respectively. The length of silique of mutant line in both ecotype backgrounds is much shorter than that of WT plants. However, antisense transformed plant did not show club shaped phenotype.

3.3.2 Genetic Analysis --- Segregation of T₂ plants

The progeny of the transgenic lines in the T₂ generation were tested for resistance to kanamycin to determine whether the plants contained the introduced neomycin phosphotransferase gene, which confers Kan^R, and see if the segregation of transgenic plants fit into Mendelian law (Table 3.1). Before presenting the results of these tests on the transgenic lines produced here, a summary of the possible outcomes is presented. Assuming that primary transformant (T₁) contains a single copy of the transgene and assuming Mendelian inheritance, homozygous Kan^R progeny for the transgene will account 25%, and 50% would be heterozygous. Kan^S progeny will account for another 25%. Since kanamycin resistance is a dominant trait, plants which are both homozygous and heterozygous for this gene are resistant to kanamycin. So, progeny would be 75% Kan^R, if transgenic plant genome has one insert gene. On the other hand, more than 75% of the progeny would be Kan^R if the transgenic plant genome has more than one insert genes. The percentage of Kan^S plants (WT) were calculated, and it had been found that the percentage of Kan^S of the following lines, Anti-L2, 3, 9, 10, 11, 16, 19, 20, 22, 24, 30, 31, 37, 38, 39, 41, was around 25%, following typical Mendelian inheritance. And it is likely these plants contain a single copy of the transgene in their genome. On the other hand, the Kan^S plant rate (%) in some transgenic lines, for instance, Anti-L1, 5, 8, 32, were less than 5%, the transformants displayed non-Mendelian segregation ratios with a very high proportion of Kan^R plants among the progeny, suggesting these transgenic lines contain multiple, unlinked copies of transgenes.

Table 3. 1 Screening seeds of T₂ transgenic plants on MS solid media with Kanamycin.

Lines No.	Number of Total plants	Number of Kan ^s plants	Number of Kan ^H Plants	Percentage of Kan ^s plants (%)
Anti-L1^	65	3	62	4.62
Anti-L2#	60	14	46	23.33
Anti-L3#	63	11	52	17.46
Anti-L5^	65	1	64	1.54
Anti-L8*^	62	2	60	3.23
Anti-L9*#	68	14	54	20.59
Anti-L10#	54	10	44	18.52
Anti-L11#	57	12	45	21.05
Anti-L12	32	2	30	6.25
Anti-L13	59	18	41	30.51
Anti-L14	79	3	76	3.80
Anti-L15	46	4	42	8.70
Anti-L16*#	71	14	57	19.72
Anti-L18*	59	8	51	13.56
Anti-L19#	65	15	50	23.08
Anti-L20#	44	9	35	20.45
Anti-L21	49	4	45	8.16
Anti-L22#	61	20	41	32.79
Anti-L23*	61	0	61	0.00
Anti-L24#	56	10	46	17.86
Anti-L25	35	3	32	8.57
Anti-L26	51	8	43	15.69
Anti-L27	65	0	65	0.00
Anti-L28	44	6	38	13.64
Anti-L29	53	9	44	16.98
Anti-L30*#	46	11	35	23.91
Anti-L31#	63	17	46	26.98
Anti-L32*^	59	2	57	3.39
Anti-L33	56	9	47	16.07
Anti-L34	48	9	39	18.75
Anti-L36	32	4	28	12.50
Anti-L37#	53	14	39	26.42
Anti-L38#	52	13	39	25.00
Anti-L39#	44	8	36	18.18
Anti-L40	63	26	37	41.27
Anti-L41#	21	4	17	19.05

Notes: * showed mutant phenotype; # showed Mendelian segregation pattern; ^

showed non-Mendelian segregation pattern.

3.3.3 Homozygous Mutants

To speed up the growth of transgenic plants, six seeds were chosen randomly from each line in T_2 generation, and grown in soil in the long-day condition greenhouse. Seeds (T_3) from 6 different plants within the same line were harvested individually and screened on MS with kanamycin. The findings were that the homozygous lines and WT plants were segregated out (Figure 3.5C). The homozygous mutants are stable and good for future research without any selection.

3.3.4 Morphological Analysis of Antisense Transformed T_1 Plants vs. WT Plants.

To determine if there are any morphological differences between antisense transformed plants and WT plants in addition to the difference of petal number, the height of plant, length of silique, weight of seeds per plant, and diameter of stem were chosen to be measured.

The average value of each chosen parameter and its corresponding standard deviation is summarized in Table 3.2. And the height of the plants, length of siliques, weight of seeds (per plant) and diameter of stems against different types of plants were plotted in Figure 3.8.

Table 3. 2 Average of each measurement of WT and T₁ antisense transformed plant.

Lines	Height (cm)	Length of Siliques (cm)	seed weight(g)	Diameter of stem (mm)	
				15cm above soil	3 cm above soil
WT plant (n=16)	43.56±6.62	1.33±0.11	0.29±0.10	0.73±0.11	0.99±0.14
Transgenic plant (n=41)	49.68±9.09	1.16±0.28	0.16±0.07	0.89±0.17	1.29±0.23

The results showed that transgenic plants were approximately 6 cm higher than WT plants, and this difference was significant ($P<0.01$); in terms of the length of silique, antisense transformed plants were significantly shorter than that of WT plants ($P<0.01$) (Figure 3.7K), but antisense transformed plants did not display the club-shaped phenotype that is found in the *clv1-1* mutant in the Landsburg background. The weight of the seed in the transgenic plants was much lower than WT ($P<0.01$); and the diameter of the stem of transgenic plants was bigger than WT plants ($P<0.01$), indicating that transgenic plants produced more secondary growth than WT plants did. Actually, the diameter of stems will be studied in greater detail in the following section to ascertain whether this gene has an effect on the amount of wood production.

Figure 3. 8 The comparisons of WT plants and T₁ antisense transformed plants.

A: Plant height (cm)

B: Silique length (cm)

C: Seed weight (mg)

D: Stem diameter (mm)

A

B

C

D

3.4 Molecular Analysis of T₃ Progeny

3.4.1 Confirmed Transgenes in Transgenic Lines Using PCR

To confirm there was a transgene in the transgenic plants, the PCR was carried out by using genomic DNA from T₃ generation as the template DNA, and the primers that were designed for these reactions flanked 35S promoter region and NOS region. The empty pSOL2 vector was used as the positive control; its PCR product would be much shorter (437 bp) because the transgene would not be present. The results (Figure 3.9A) showed that there were transgenes in the four lines that were tested, and PCR product was 1042bp. Thus transgene was present, being 605 bp in length.

3.4.2 Southern Blot

The number of copies of T-DNA in the genomes of the transformed plants was investigated by Southern blot analysis. Genomic DNA was isolated from T₃ progeny and digested with *EcoRI*. It was then resolved by electrophoresis and blotted. The blots were probed with a 640 bp portion of the 35S promoter region, which detects the T-DNA-plant right border fragment. There are no restriction sites for the above restriction enzyme within 35S promoter region. Therefore, the number of hybridizing bands in the genomic DNA of each progeny should indicate the number of T-DNA insertions.

The results (Figure 3.9B) showed that there were more than one insert in Anti_L23, while there was one insert in Anti_L30 and Co_L7. This finding is consistent with the results of screening seeds of T₂ transgenic plants on MS solid media with Kanamycin (Table 3.1). In summary, the transformed plants could be

grouped into two classes. One class is composed of transgenic plants that have more than one insert in their genomes, they turned out to have a lower percentage of Kan^S (less than 10%). The other class, plants with only one insert in their genome have roughly 25% Kan^S plants.

Figure 3. 9 Confirmation of transgenes in the transformed plants

A) Confirmation of transgenes in the transformed plants by running a PCR.

The forward primer was designed based on the 35S promoter region and the reverse primer was designed according to NOS region. The expected PCR product should contain the antisense or sense *CLV1* region. The empty pSOL2 vector was used as a positive control.

B) Southern-blot analysis (cut by *EcoRI*) of transgenic *Arabidopsis* lines.

Genomic DNA (10 µg per lane) from 13 independently transformed lines and from an untransformed line was digested by *EcoRI*. DNA restriction fragments were separated on a 1% (w/v) agarose gel, transferred to nylon membranes, and hybridized with the ³²P labeled, 35S promoter fragment probe. The analysis demonstrated that each of lines was the result of one to three integration events. Numbers to the left indicate molecular mass standards in base pair.

A

B

3.4.3 RT-PCR to Detect the Expression of *CLV1*

3.4.3.1 *CLV1* Expression Pattern in Different Organs

To determine in which organs *CLV1* gene is expressed, total RNA was isolated from the *Arabidopsis* root, hypocotyl, stem, leaf, vegetative shoot meristem and the flower. The corresponding cDNA was subsequently synthesized and RT-PCR was performed. *CLV1* probe DNA (1189 bp) was used as template DNA in the control lane. The result (Figure 3.10A) showed that *CLV1* was expressed in the flower and the shoot meristem, while no PCR products were found in the root, hypocotyl, stem or leaf. The results are consistent with the finding of Clark *et al.* (1997).

3.4.3.2 *CLV1* is Expressed in the old Hypocotyl Grown under the Short-day Conditions.

To further explore if the *CLV1* gene is expressed in the vascular meristem of hypocotyl, total hypocotyl RNA was isolated from two-month-old plant grown under the short day conditions in order to run RT-PCR. To rule out the possibility of genomic DNA contamination in the RNA samples, the hypocotyl RNA itself was treated as 'template' as a positive control at the same time. It has shown that *CLV1* is expressed in 2-month-old hypocotyl (Figure 3.10B), indicating *CLV1* gene may involve in the growth of the vascular meristem of the hypocotyl, regulating the wood formation directly.

Figure 3. 10 The expression pattern of *CLV1* gene

A) *CLV1* was only expressed in the vegetative shoot meristem, floral meristem and flowers. RNA isolated from root, hypocotyl, stem, leaf, shoot meristem and flower was converted to cDNA to carry out RT-PCR. The RNA was extracted from 30 day-old plants.

B) The *CLV1* gene was expressed in the 2 month-old hypocotyl. RNA was treated as 'template DNA' in lane 1, 2 and 3. The template DNA was cDNA synthesized from hypocotyl RNA in the lanes 5, 6, and 7.

A.

B.

3.4.4 Northern Blot

To determine the transcription level of antisensed *CLV1* gene in the antisense-transformed lines, a Northern blot was performed using *CLV1* as a probe (Figure 3.11A). The reason that Anti_L8, L16, L27 and L32 lines were chosen are: 1) these lines showed the *clv1* phenotype (extra petal), suggesting they were strong mutant lines; 2) lines with different transgene copy numbers could shed light on the transcription level of the transgene. The results showed that the transgene was highly expressed in the transformed line, and it was noted that the lines with a multi-copy number transgene (L32) had higher transcriptional levels than the line with a single copy number transgene (L16) (Figure 3.11C). In the control lanes, no transgene signal was found in WT flower and hypocotyl. More importantly, the *CLV1* gene was more highly expressed higher in the WT flower and hypocotyl than that of transformed lines. This suggests that the endogenous *CLV1* transcript in transformed lines was inhibited by the antisense *CLV1* gene (Figure 3.11B).

Figure 3. 11 Northern blot analysis.

- A) Northern blot. *CLV1* mRNA levels in the transgenic *Arabidopsis* flower was examined by northern blotting. Total RNA (10 µg) was prepared from flowers of WT plant and from four mutant lines; another 10 µg was isolated from WT hypocotyl grown under the short day conditions. Blot was hybridized to the radioactively labeled, *CLV1* coding region. Equivalent loading of RNA was assessed by a pre-run gel. Arrows and labels indicate the position and size of the transcripts.
- B) *CLV1* expression levels. Endogenous *CLV1* transcript in transformed lines was inhibited by the antisense *CLV1* gene.
- C) Antisense *CLV1* expression levels. Multi-copy number transgene line(L32) had higher transcriptional levels.

A

B

C

3.5 Cellular Analysis of T₄ Progenies

3.5.1 Comparison of Radius of Xylem and Phloem/Cortex in Hypocotyl between WT and Mutant Lines

The xylem and phloem/cortex are easily distinguished in a hand-section stained with the Toluidine blue O (pH, 4.4). The xylem part turned out to be blue since the mature vessels and fibers have thick lignified cell walls; the phloem/cortex part is pink, since there are fewer cells with secondary walls here (Figure 3.12 C, D).

Ten *CLV1* antisensed lines, one *CLV1* sensed line and one WT line were chosen for measuring the total xylem radius, and total hypocotyl radius, each line was composed of more than 6 plants. The phloem/cortex radius could be determined by total hypocotyl radius minus total xylem radius. The results are shown in the following Table 3.3, and graphically represented in Figure 3.13A.

Table 3. 3 Radius (μm) of Xylem and Phloem/Cortex in Hypocotyl between WT and Mutant Lines.

Lines	Xylem Radius	Phloem/Cortex Radius
WT	359.06 ±31.53	380.77±24.13
Co_L7	470.97±30.01	467.97±39.44
Anti_L8	504.99±9.52	441.97±33.55
Anti_L9	516.54±6.45	652.81±48.32
Anti_L10	570.00±9.54	450.38±16.75
Anti_L16	538.25±28.45	503.92±66.15
Anti_L18	425.00±16.48	406.67±15.20
Anti_L23	496.15±27.25	464.55±25.12
Anti_L27	560.00±19.66	663.33±36.67
Anti_L30	396.00±13.27	536.00±58.02
Anti_L32	514.00±14.70	730.00±24.90
Anti_L40	505.00±8.24	436.25±43.05

It was shown that both xylem radius and phloem/cortex radius are found to be greater than that of wild type plant. For instance, xylem radius of Anti_L10 line

has been increased by 58.75% compared to WT line. Generally speaking, the average xylem radius of 10 antisensed lines is increased by 39.98% compared to wild type line, and the average phloem/cortex radius is increased by 38.82%. T-test results show that xylem radius of all antisense transformed lines except for Anti_L18 and L30 are significant greater than that WT plants ($P < 0.01$), and phloem/cortex radius of Anti_L9, 27, 30, and 32 are bigger than that of WT plants ($P < 0.05$). These data showed compelling evidence that both xylem and phloem formation is negatively regulated by CLV1.

3.5.2. Comparison of Area (μm^2) of Xylem and Phloem/Cortex in Hypocotyl between WT and Mutant Lines

Radius is a linear measurement, and does not fully represent the significance of growth in 2-dimensions. So the surface area of circular cross sections was calculated as seen in Table 3.4, and graphically represented in Figure 3.13B.

Table 3. 4 Area (μm^2) of Xylem and Phloem/Cortex in Hypocotyl between WT and Mutant Lines.

Lines	Xylem Area	Phloem/Cortex Area
WT	433121.26 $\pm$ 77332.32	1357614.25 $\pm$ 163296.81
Co_L7	708145.45 $\pm$ 91316.96	2079815.08 $\pm$ 213802.44
Anti_L8	802582.57 $\pm$ 29596.34	2025071.89 $\pm$ 177105.76
Anti_L9	838880.15 $\pm$ 21073.31	3487777.25 $\pm$ 338744.52
Anti_L10	1021835.64 $\pm$ 34606.84	2255955.59 $\pm$ 125106.27
Anti_L16	920321.19 $\pm$ 95023.07	2573766.88 $\pm$ 460630.81
Anti_L18	571717.50 $\pm$ 46324.69	1612265.35 $\pm$ 101151.09
Anti_L23	785011.92 $\pm$ 91696.90	2134049.14 $\pm$ 156753.99
Anti_L27	991277.20 $\pm$ 66107.19	3755774.02 $\pm$ 322777.64
Anti_L30	494863.67 $\pm$ 32371.58	2290975.03 $\pm$ 360131.29
Anti_L32	832710.55 $\pm$ 47593.17	4037135.06 $\pm$ 193960.20
Anti_L40	802676.92 $\pm$ 26144.47	2021497.06 $\pm$ 283791.52

From this calculation, a 38.98% increase in xylem radius is equivalent to an 86.13% increase in cross-sectional area in antisensed mutant lines, and a 38.82% increase in phloem/cortex is equivalent to a 92.94% increase in the antisensed mutant lines.

These results indicate that disruption in the *CLV1* locus causes an increase in the radius of xylem and phloem, leading to a significant increase of wood formation.

Figure 3. 12 The transverse stem and hypocotyl sections of WT and antisensed transgenic plant.

A, B) A hand-sectioned, Toluidine blue O stained stem cross section. Anti_L32 (B) has 20 bundles while the WT plant (A) has 13 bundles.

C, D) A hand-sectioned, Toluidine blue O stained hypocotyl cross-section, showing the mutant line (Anti_L9) (D) on the right, and the wild type (C) on the left. The increased diameter of the hypocotyls is due to both increased xylem and increased phloem/cortex development.

Figure 3. 13 Comparison of xylem and phloem/cortex radius and area of cross section between WT and mutant lines.

Each line was tested on more than six plants. Analysis was performed in duplicate and results were presented as the mean with standard error.

A) Radius of hypocotyl cross section

B) Area of hypocotyl cross section

A

Comparison of the Xylem and Phloem/cortex Radius of Cross Section among WT and Mutant Lines

B

Comparison of Xylem and Phloem/cortex Areas among WT and Mutant Cross Sections

3.5.3 Comparison of Radius of Stem (mm) between WT and Mutant Lines

To test if there was also a change in the activity of the vascular and fascicular cambium of the stem, the radius of stem and the number of bundles was measured at the base and number of bundles was counted based on the cross sections as seen in Table 3.5, and graphically represented in Figure 3.24.

Table 3. 5 The radius (mm) of stems of WT and transgenic plants.

Lines	Radius (mm)	Increase (%)
WT	0.53 ± 0.02	
Co_L7	0.66 ± 0.01	24.53
Anti_L8	0.70 ± 0.02	32.08
Anti_L9	0.78 ± 0.01	47.17
Anti_L10	0.59 ± 0.02	11.32
Anti_L16	0.72 ± 0.01	35.85
Anti_L18	0.87 ± 0.01	64.15
Anti_L23	0.64 ± 0.01	20.75
Anti_L27	0.69 ± 0.04	30.19
Anti_L30	0.65 ± 0.01	22.64
Anti_L32	1.05 ± 0.16	98.11
Anti_L40	0.62 ± 0.02	16.98

It can be seen from Figure 3.25 that WT plant has 13 bundles, but Anti_L32 has 20 bundles in cross section of stem. And radius of Anti_L32 stem is twice as much as that of WT plant. This increased radius in these antisense lines has a 37.92% increase compared to WT plants. T-test results show that stem radius of Anti_L8, 9, 16, 18, 23, and 30 are significant greater than that of WT plants ($P<0.01$), stem radius of Anti_L40 is bigger than that of WT plants ($P<0.05$), while Anti_L27 does not show any difference compared to stem size of WT plants.

Taken together, these results show that the *CLV1* gene was successfully downregulated in transgenic plants, and the phenotype was typical of a strong

allele of the *clv1* mutant. As a result of the loss of CLV1 the floral meristems were altered as evidenced by the change in the number of petals. The loss of CLV1 also resulted in an increase in cambial meristem activity, which caused an increase in both xylem and phloem in the secondarily thickened hypocotyls. These results indicate that CLV1 is also a negative regulator of the vascular cambium.

Figure 3. 14 Comparison of stem radius of cross section between WT and mutant lines.

Each line was tested on more than six plants. Analysis was performed in duplicate and results are presented as the mean with standard error.

Comparisom of Stem Radius among WT and Mutant Lines

4. Discussion

Our results have shown that antisense is a good technique to downregulate *CLV1* expression. Eight out of 41 transformed *Arabidopsis* lines had extra petals in the flower, which is typical of a strong *clv1*-like phenotype. The results of Southern blot analysis indicated that at least one transgene was integrated in *Arabidopsis* genome for each line. And northern blot analysis of the transgenic lines showed that the transgene transcript was highly expressed in the flowers of transformed lines while the level of endogenous *CLV1* transcripts were greatly reduced. In northern blots of wild-type plants, the *CLV1* transcript was found in the 2 month-old hypocotyl. Since this is a tissue that is undergoing extensive secondary growth, we hypothesize that CLV1 may be directly involved in the regulation of the vascular cambium. This thesis investigates the role of CLV1 in the development of the vascular cambium through the downregulation of *CLV1* gene expression by antisense. Consistent with the *clv1* mutant, the antisense *CLV1* plants had significantly more secondary growth, suggesting that CLV1 is a negative regulator of the vascular cambium.

Based on this work, it is reasonable to expect that a similar strategy to downregulate *CLV1* expression in the stem of a tree would also cause an increase in xylem formation. The potential application of this strategy to trees is being explored.

4.1. Identifying *clv1*-like Phenotypes in Mutant Lines

It has previously been shown that the *clv1* floral meristem can be twice as tall as the WT floral meristem at the earliest stage of organ initiation (Clark *et al.*, 1997). However, dissecting the meristem out of an *Arabidopsis* plant and measuring the size by scanning electron microscopy is far too time consuming and labor intensive to be used as a screen for analyzing transgenic plants. Luckily, the *CLV1* mutation has an additional effect on the development of the floral meristem that is much easier to detect than the effect on the development of the SAM, which is the formation of extra petals on the flower. So extra petals were chosen as a preliminary indicator of a *clv1*-like phenotype. Based on this initial screen it is possible that some transgenic lines with weaker floral phenotypes, but equally strong vascular cambium phenotypes, were missed, but because of the large number of plants to be analyzed, it was essential to restrict the phenotypic analysis to the most promising lines. We have shown that the number of petals in the flower is a good indicator of a strong *clv1*-like phenotype in the transgenic plants.

In our experiments, *clv1* phenotypes similar to the strong mutant alleles have been mimicked, thus indicating antisense was strong enough to interfere with the endogenous *CLV1* transcript and prevent the production of enough *CLV1* to negatively regulate meristems. All transgenic lines were advanced to the T₃ generation and homozygous lines were chosen where possible since it has been shown that silencing occurs much more efficiently when T-DNA is in a homozygous state (Elmayan *et al.*, 1996). Only 19.51% of the antisense

transformed plants displayed a strong *clv1* mutant phenotype. These strong alleles are, *clv1-4* and *clv1-8*, which both contain missense mutations in the LRR domain. Our current understanding of how antisense works is that the antisense transcript binds to the endogenous transcript and prevents translation, either by physically interfering with ribosome assembly on the transcript or by causing the double stranded RNA to be targeted for degradation. Because we were able to mimic strong *clv1*-like phenotypes in these transgenic plants, it appears that the antisense transcript was produced in the correct tissues and at a high enough level to interfere with the endogenous *CLV1* transcript.

The transgenic lines showed the broad range of flower phenotypes. For example, Anti_L22 showed 3 petals, while Anti_L9, 16, 18, 23, and 32 had extra petals. Several other lines had no decreased or increased petal number but their flower shape was deformed such as the angle of the petals compared to the rest of the flower. This variation in phenotype from one line to the next is not unexpected, since transgene expression is known to be affected by copy number and insertion location. Both of these factors can alter the level of expression of the transgene and ultimately affect its impact on endogenous *CLV1* expression levels. (Palauqui *et al.*, 1998).

4.2.The growth of Vascular meristems is Negatively Regulated by CLV1

Our results have shown that the growth of vascular cambium and the vascular meristem are both negatively regulated by CLV1. In the primary stem, WT plants have 13 vascular bundles while the Anti_L32 has 20 bundles. This

also resulted in an increased radius in the 10 antisense lines of 37.92% compared to WT plants. This is consistent with previous findings that showed that bundle number is proportional to meristem size. The increase of bundle number and stem radius could result from a direct consequence of the increase in meristem size or indirect consequence of the increase in organ number associated with larger meristem (Doil *et al.*, 1998). Another distinct feature of antisensed plants is that they have sturdy stems compared to WT plants, likely resulting from the increased number of lignified vascular bundles.

The secondary growth in the hypocotyl of *Arabidopsis* made it possible to also investigate the effect of CLV1 on a vascular cambium. Because of the similarity in structure of the secondarily-thickened hypocotyl and the stem of trees, it is expected that these results will be the most applicable to wood formation (Chaffey *et al.*, 2002). Our results have shown that both xylem radius and phloem/cortex radius are found to be greater than that of wild type plants. Over all, the average xylem radius of 10 antisensed lines is increased by 39.98% compared to wild type, and the average phloem/cortex radius is increased by 38.82%. Accordingly, the area of xylem and phloem/cortex are increased by 86.13% and 92.94% respectively.

From the results presented in this thesis, it is not known when *CLV1* expression is turned on in the vascular cambium, but *CLV1* mRNA was detected in two month-old hypocotyls when both secondary xylem and phloem are produced. To precisely locate the *CLV1* gene expression pattern in the hypocotyl, future research should include *In situ* hybridization on hypocotyls of

various stages, especially during the induction and maintenance of the vascular cambium.

CLV1 is the first example of a negative regulator of the vascular meristem, and it appears to affect xylem and phloem equally. There have been several positive regulators of vascular meristems identified. The first regulator that was identified was *WOL* (*WOODEN LEG*), a recessive mutation in the *WOL* gene results in reduced proliferation of procambial cells, deformed xylem organization, and absence of phloem cells within root vascular tissue (Scheres *et al.*, 1995). The second are two tissue specific genes regulating the growth of phloem and xylem separately (Graaff *et al.*, 2002). *VAS* positively regulates growth of phloem cambial and pericycle cells while *LEP* can cause a specific increase in the number of xylem cells. The third positive regulator *ATHB-8* has been found to be involved with the growth of vascular meristem (Baima *et al.*, 2001). Originally, *ATHB-8* was found to participate in a positive feedback loop of auxin signaling transduction, which in turn positively regulate the activity of procambial and cambial cells to differentiate. The last one is *IFL1* (*INTERFASCICULAR FIBERLESS1*), which is necessary for lateral meristem initiation and normal organ development (Otsuga *et al.*, 2001). *CLV1* is the first positive regulator of the vascular meristem that has been shown to also affect the fascicular cambium; all the other regulators have only been studied in primary vascular development.

4.3. Is Antisense the best Strategy for Downregulating *CLV1*?

Antisense technology has been used widely for gene function studies. The working mechanism of antisense is quite simple. Once mRNA forms a duplex with a complementary antisense RNA sequence, translation from target mRNA is blocked. The possible reasons for this are 1) the ribosome can not gain access to the nucleotides in the mRNA, or 2) duplex RNA is quickly degraded by ribonucleases in the cell.

(<http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/A/AntisenseRNA.html>).

Another gene silencing technique that has gained much attention in the last couple years is RNAi. It seems more potent than antisense technique. According to Hutvagner *et al* (2002), the current idea of how RNAi works is as follows. In the initiation step, the dsRNA (double stranded RNA), which is usually several hundred base pair long, is cleaved by enzyme Dicer that is a member of RNAase III family of ribonucleases to form 21-23 bp double stranded fragments known as siRNAs (short interfering RNAs) or guide RNAs. In the effector step, the duplex siRNAs are then unwound by a helicase activity known as a member of RISC (RNA-induced silencing complex). The last step is that the siRNA strand is complementary to the target mRNA is then used as primer by an RdRP (RNA-dependent RNA polymerase) to convert the target mRNA into dsRNA itself. So the new round of ds RNA cutting by Dicer begins.

There were several reasons why we chose an antisense rather than an RNAi approach for this project. First, antisense provides a more controllable silencing and is often not complete or as strong as RNAi (Guru, 2000). RNAi

results in a complete silencing that travels systemically throughout the plant and is reported to be 1000-fold more effective than antisense (Thakur, 2003). In our experiment, *CLV1* is expressed in many organs, such as the floral meristem, shoot meristem and the vascular cambium of secondarily thickened hypocotyls. Ultimately we would like to alter the cambial meristem without interfering with the function of the SAM or floral meristem. Because antisense does not necessarily result in a systemic silencing response, it provides an opportunity to downregulate *CLV1* in only the tissues of interest, assuming a suitable promoter could be found. Second, despite being considered a weaker strategy, antisense can also give rise to dsRNA, which is an actual inducer of DNA methylation (Thakur, 2003), involving gene silencing. Furthermore, it has been reported that antisense constructs probably also produce RNAs that feed into the dsRNA-induced degradation pathway (Stam *et al.*, 2000). Third, the RNAi response exhibits extremely stringent sequence specificity, a single base pair mismatch over the length of a siRNA is enough to block the response (Thakur, 2003). Fourth, RNAi can efficiently attack both their mismatched target sites as well as only partial complementary sequences on nontarget genes, thus leading to concern about their specificity; Fifth, RNAi does not work well with some RNA targets including highly structured RNAs, short-lived transcripts and viral RNAs having high mutation rates (www.somagenics.com/competitive.html).

Since we ultimately want to transfer the strategy used in this thesis to trees, it was important that we chose a method that has the highest potential of being specific to the vascular cambium, while still being strong enough to elicit

the required silencing. Our results show that the antisense approach is strong enough to provide silencing of CLV1 such that the transgenic plants displayed *clv1*-like phenotypes. Future research will focus on identifying a suitable promoter that is expressed only in the vascular cambium, so that silencing can be directed only to this tissue.

4.4 Tissue Specific Expression Promoter

CaMV 35S promoter is expressed throughout the plant and also has been shown to be active in green algae, yeast and *E. coli* (Ho *et al.*, 1999); it is the most frequently used promoter in genetically modified plants because of its widespread expression. But there are also drawbacks of using such a strong promoter with such a broad expression pattern, especially if you want to target your transgene to specific tissue or during a specific time in development. In our case, it would be ideal to have a promoter that was expressed in the vascular meristem that produces the secondary xylem and phloem. Vincient *et al* (2001) found the AtEm1 promoter is highly active in the vascular tissues of the embryo, also active in petals and sepals; another MERISTEM LAYER1 promoter (Sessions *et al.*, 1999) specifies expression in meristems. The most promising promoters for xylem-specific expression in *Arabidopsis* are XCP1 and XCP2 (Beers *et al.*, 2001). These promoters direct expression in protoxylem, metaxylem and secondary xylem tracheary elements and therefore would be good candidate promoters for future research.

Eventually, our finding in *Arabidopsis* will be applied to poplar, so choosing a vascular tissue specific expression promoter is more practical. Gray-Mitsumune *et al.* (1997) found APL2 promoter was a good candidate for wood formation research.

4.5. Future Research Work

Our work has shown that CLV1 is negatively regulating the growth of the vascular cambium in *Arabidopsis*. We have shown that *CLV1* transcripts accumulate in 2-month old hypocotyls and downregulating *CLV1* results in an increase of both xylem and phloem. A more detailed analysis of CLV1 expression may provide more insight into its role in cambial meristem development, and therefore *In situ* hybridizations at specific timepoints in development would be valuable.

Our results have also shown that the *clv1*-like phenotypes can be duplicated by antisense, making this strategy possible for other plants such as trees. It is reasonable to expect that CLV1 is also working in the cambium of trees, and work is underway right now to downregulate *CLV1* in poplar. We have prepared the antisense construct for a *CLV1*-like clone in poplar and it is currently being tested in poplar. Additionally, two *CLV1*-like clones have been identified in a poplar EST library (Goran Sandberg, personal communication). Microarray analysis has revealed that one clone is expressed in the xylem and the other in the phloem, suggesting CLV1 plays an important role in cambium

development in poplar, but because there appears to be two, it might be more complicated than in *Arabidopsis*.

One of the advantages of trees is the ability to isolate large amounts of pure xylem and phloem tissue. In fact, the Swedish lab can isolate RNA from specific stages of secondary xylem development (Hertzberg *et al.*, 2001), making it possible to see expression at a level that would be impossible in *Arabidopsis*.

Future work will look specifically at the expression of *CLV1* in poplar, at a variety of growth regimes that are known to up and down regulate the activity of the vascular cambium. Assuming that the vascular cambium is as complicated as the shoot apical meristem, we can expect that there will be many other genes that regulate the cambium. Some candidates might be the other proteins that regulate the SAM, such as STM, CLV2, CLV3, WUS, POL, but it is also possible that there are other proteins involved. Future microarray analysis of poplar cambial tissues will help to investigate the genes expressed in the cambium. Our research has been supported by Genome Canada and part of the project is to produce poplar microarray. As the antisensed *CLV1* plants are produced, these microarrays will be applied to these trees to reveal alterations in the expression of the potential regulators of the vascular cambium. Finally, this work has provided the first piece of evidence on how the cambium is regulated, and the tools produced in this thesis will be used to further understand how the cambium is regulated in both *Arabidopsis* and poplar.

5. References

- An, G., B.D. Watson and C.C. Chiang.** (1986). Transformation of Tobacco *Nicotiana ssp. tomato Lycopersicon esculentum*, potato *Solanum tuberosum* and *Arabidopsis thaliana* using binary Ti vector system. *Plant Physiol.*(Bethesda). **81**: 301-305.
- Arioli T, Peng L, Betzner AS, Burn J, Wittke W, Herth W, Camilleri C, Hofte H, Plazinski J, Birch R, Cork A, Glover J, Redmond J, Willoamson RE.** (1998). Molecular analysis of cellulose biosynthesis in *Arabidopsis*. *Science*. **279**: 717-20.
- Azpiroz-Leehan R, Feldmann KA.** (1997). T-DNA insertion mutagenesis in *Arabidopsis*: going back and forth. *Trends Genet*. **13**: 152-156.
- Baima S., Possenti M., Matteucci A., Wisman E., Altamura M.M., Ruberti I., and morelli G.** 2001. The *Arabidopsis* ATHB-8 HD-Zip protein acts as a differentiation-promoting transcription factor of the vascular meristems. *Plant Physiology*. **126**: 643-655.
- Bechtold N, Ellis J, Pelletier G.** (1993). *In Planta* Agrobacterium-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C R Acad Sci Paris Life Sci*. **316**: 1194-1199.
- Beers E.P., and Zhao C.Z.**(2001). *Arabidopsis* as a model for investigating gene activity and function in vascular tissues. *Molecular Breeding of Woody Plants*. P43-52.
- Bent, AF.** (2000). *Arabidopsis* in planta transformation. Uses, mechanisms, and prospects for transformation of other species. *Plant Physiol*. **124**: 1540-1547.
- Birch RG.** (1997). Plant transformation: problems and strategies for practical application. *Annu Rev Plant Physiol Plant Mol Biol*. **48**: 297-326.
- Bowman JL, Eshed Y.** (2000). Formation and maintenance of the shoot apical meristem. *Trends in Plant Science*. **5**: 110-115.
- Brand U., Fletcher J.C., Hobe M., Meyerowitz E.M and Simon, R.** (2000). Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by CLV3 activity. *Science*. **289**: 617-619.
- Brantl.** (2002). Antisense-RNA regulation and RNA interference. *Biochimica et Biophysica Acta*. **1575**: 15-25.
- Bruening G.** (1998). Plant gene silencing regularized. *Proc. Natl. Acad. Sci. USA*. **95**: 13349–13351.

Busse JS, Evert RF. (1999). Vascular differentiation and transition in the seedling of *Arabidopsis thaliana*. *Int. J. Plant Sci.* **160**:241-251.

Chaffey N, Cholewa E, Regan S, Sundberg B. (2002). Secondary xylem development in *Arabidopsis*: a model for wood formation. *Physiol Plant.* **114**: 594-600.

Chang S-S, Park S-K, Nam H-G. (1990). Transformation of *Arabidopsis* by *Agrobacterium* inoculation on wounds. In: *Abstracts of the Fourth International Conference on Arabidopsis Research. Vienna.* pp28.

Clark, SE. (2001a). Cell signaling at the shoot meristem. *Nature Reviews Mol. Cell Biol.* **2**: 276-284.

Clark, SE. (2001b). Meristems: Start your signaling. *Current Opinion in Plant Biology.* **4**: 28-32.

Clark SE, Williams RW and Meyerowitz EM. (1997). The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. *Cell.* **89**: 575-585.

Clark SE, Running MP, Meyerowitz EM. (1995). *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. *Development.* **121**: 2057-67.

Clark SE, Running MP, Meyerowitz EM. (1993). *CLAVATA1*, a regulator of meristem and flower development in *Arabidopsis*. *Development.* **119**:397-418.

Clarke MC, Wei W, Lindsey K. (1992). High frequency transformation of *Arabidopsis thaliana* by *Agrobacterium tumefaciens*. *Plant Mol Bio Rep.* **10**: 178-189.

Clough SJ, Bent AF. (1998). Floral dip: simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**:735-743.

Cutter, E. G. (1965). Recent experimental studies of the shoot apex and shoot morphogenesis. *Bot. Rev.* **31**: 7-113.

Desfeux C, Clough SJ, Bent AF. (2000). Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the *Arabidopsis* floral-dip method. *Plant Physiol.* **123**: 895-904.

Dievart A, Dalal M, Tax FE, Lacey AD, Huttly A, Li J, and Clark SE. (2003). *CLAVATA1* dominant-negative alleles reveal functional overlap between multiple

receptor kinase that regulate meristem and organ development. *The Plant Cell*. **15**: 1198-1211.

Doil LM, Jeong SH. and Clark SE. (1998). The role of meristem structure and organ formation on vascular patterning. *9th International Conference on Arabidopsis Research*.

Elmayan T., H. Vaucheret (1996) Single copies of a strongly-expressed 35S-driven transgene undergo post-transcriptional silencing. *Plant J* **9**:787-797.

Ewen S, Pusztai A. (1999). Effect of diets containing genetically modified potatoes expressing *Galanthus nivalis* lectin on rat small intestine. *The Lancet*. **354**: 1353-1354.

Feldmann KA, Marks MD. (1987). *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*. *Mol Gen Genet* . **208**: 1-9.

Fletcher JC, Brand U, Running MP, Simon R, and Meyerowitz EM. (1999). Signaling of cell fate decision by *CLAVATA3* in *Arabidopsis* shoot meristems. *Science*. **283**: 1911-1914.

Flecher, JC. (2002). Shoot and floral meristem maintenance in *Arabidopsis*. *Annu. Rev. Plant. Biol.* **53**:45-66.

Franklin GL. (1945). Preparation of thin sections of synthetic resins and wood-resin composition, and a new macerating method for wood. *Nature*. **155**:51.

Fukuda H. (1996). Xylogenesis: initiation, progression, and cell death. *Ann. Rev. Plant Physio. Plant Mol. Biol.* **47**: 299-325.

Gendreau E, Traas J, Desmos T, Grandjean O, Caboche M, Hofe H. (1997). Cellular basis of hypocotyl growth in *Arabidopsis thaliana*. *Plant Physiol.* **114**: 295-305.

Graaff EVD, Hooykaas PJJ and Keller B. (2002). Activation tagging of the two closely linked genes LEP and VAS independently affects vascular cell number. *The Plant Journal*. **32**: 819-830.

Gray-Mitsumune M, Molitor EK, Douglas CJ, and Carlson J. (1997). Vascular specific expression of the poplar PAL2-GUS fusion gene in transgenic aspen. *Proceedings of the PD-AAAS*. June 22 - 25, 1997

Guru, T. (2000). A silence that speaks volumes. *Nature*. **404**; 804-808.

Han, K-H. (2001). Molecular biology of secondary growth. *J. Plant Biotechnology*. **3**:45-57.

Hansen G, Wright MS. (1999). Recent advances in transformation of plants. *Trends Plant Sci.* **4**: 226-231.

Hertzberg M, Aspeborg H, Schrader J, Andersson A, Erlandsson R, Blomqvist K, Bhalerao R, Uhlen M, Teeri TT, Lundeberg J, Sundberg B, Nilsson P, Sandberg G. (2001). A transcriptional roadmap to wood formation. *Proc Natl Acad Sci U S A.* **98**: 14732-7.

Ho MW, Ryan A, Cummins, J. (1999) Cauliflower mosaic viral promoter "A recipe for disaster? " *Microbial Ecology in Health and Disease.* **11**: 194-197.

Hooykaas PJJ, and Schilperoot RA (1992). *Agrobacterium* and plant genetic engineering. *Plant Mol. Biol.* **19**: 15-38.

Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers, SG, and Fraley RT. (1985). A simple and general method for transferring genes into plants. *Science.* **227**: 1229-1231.

Hutvagner G and Zamore PD. (2002). RNAi: nature abhors a double-strand. *Current Opinion in genetics and Development.* **12**: 225-232.

Jenik PD and Irish VF. (2000). Regulation of cell proliferation patterns by homeotic genes during *Arabidopsis* floral development. *Development.* **127**: 1267-1276.

Jeong S, Trotochaud AE and Clark SE. (1999). The *Arabidopsis* CLAVATA2 gene encodes a receptor-like protein required for stability of the CLAVATA1 receptor-like kinase. *Plant Cell.* **11**: 1925-1933.

Kayes JM, Clark SE. (1998). CLAVATA2, a regulator of meristem and organ development in *Arabidopsis*. *Development.* **125**: 3843-51.

Katavic V., Haughn G W., Reed D., Martin M., Kunst L. (1994). In Planta transformation of *Arabidopsis thaliana*. *Mol Gen Genet.* **245**: 363-370.

Koornneef M, Van Eden J, hanhart CJ, Stam P, Braaaksma FJ and Feenstra WJ. (1983). Linkage map of *Arabidopsis thaliana*. *J. Hered.* **74**. 265-272.

Krisan PJ, Young JC, Sussman MR. (1999). T-DNA as an insertional mutagen in *Arabidopsis*. *Plant Cell.* **11**: 2283-2290.

Laux T, Mayer KFX, Berger J and Jurgens G. (1996). The *WUSCHEL* gene is required for shoot and floral meristem integrity in *Arabidopsis*. *Development.* **122**: 87-96.

Lenhard M. and Laux T. (2003). Stem cell homeostasis in the Arabidopsis shoot meristem is regulated by intercellular movement of CLAVATA3 and its sequestration by CLAVATA1. *Development*. **130**: 3163-3173.

Leyser HMO. and Furrer IJ. (1992). Characterizations of three shoot apical meristem mutants of *Arabidopsis thaliana*. *Development* **116**: 397-403.

Lev-Yadun S. (1997). Fibers and fibre-sclereids in wild-type *Arabidopsis thaliana*. *Annals. Bot.* **80**: 125-129.

Lev-Yadun S. (2001). *Arabidopsis thaliana* a new crop? *Nature Biotechnology*. **19**: 95.

Mayer K. L. (1998). Role of *WUSCHEL* in regulating stem cell fate in the *Arabidopsis* shoot meristem. *Cell*. **95**: 805-815.

Mckelvie AD. (1962). A list of mutant genes in *Arabidopsis thaliana*. *Radiation Botany*. **1**: 233-241.

Medford JI, Behringer FJ, Callos JD and Feldmann KA. (1992). Normal and abnormal development in the *Arabidopsis* vegetative shoot apex. *The Plant Cell*. **4**: 631-643.

Meinke DW, Cherry JM, Dean C, Rounsley SD, Koorneef M. (1998). *Arabidopsis thaliana*: A model plant for genome analysis. *Science*. **282**: 678-682.

Meyerowitz EM (1997). Genetic control of cell division patterns in developing plants. *Cell*. **88**: 8-308.

Meyerowitz EM. (2001). Prehistory and history of *Arabidopsis* research. *Plant Physiol.* **125**: 15-19.

Murashige T. and skoog F. (1962). A revised medium for growth and bioassays with tobacco cell cultures. *Physiol. Plant.* **15**: 473-497.

Nacry P, Camilleri C, Courtial B, Caboche M, Bouchez D. (1998). Major chromosomal rearrangements induced by T-DNA transformation in *Arabidopsis*. *Genetics*. **149**: 641-650.

Nagata M, Mori H, Tabei Y, Sato T, Hirai M. and Imaseki H. (1995). Modification of tomato fruit ripening by transformation with sense or antisense chimeric 1-aminocyclopropane-1 –carboxylate synthase genes. *Acta Hort. (ISHS)*. **394**: 231-218.

Palauqui JC, and Vaucheret H. (1998). Transgenes are dispensable for the RNA degradation step of cosuppression. *Proc. Natl. Acad. Sci. USA*. **95**: 9675-9680.

Parinov S and Sundareshan V. (2000). Functional genomics in *Arabidopsis*: large-scale insertional mutagenesis complements the genome sequencing project. *Current Opinion in Biotechnology*. **11**: 157-161.

Regan, S. (1993). The expression of adenine phosphoribosyl transferase during pollen development. *Doctoral Thesis*. University of Waterloo.

Rothstein SJ, DiMaio J, Strand M, and Rice D. (1987). Stable and heritable inhibition of the expression of nopaline synthase in tobacco expressing antisense RNA. *Proc. Natl. Acad. Sci. USA*. **84**: 8439-8443.

Running, M.P, Clark, S.E., and Meyerowitz, E.M. (1995). Confocal microscopy of the shoot apex. In *Methods in Cell Biology: Plant Cell Biology*.

Samac D. A. (2002). Promoter for constitutive and tissue-specific expression of transgenes in Alfalfa. Electric publication.

Scheres B, Di Laurenzio L, Willemsen V, Hauser M-t, Janmaat K, Weisbeek P., Benfey PN. (1995). Mutations affecting the radial organization of *Arabidopsis* root display specific defects throughout the embryonic axis. *Development*. **121**: 53-62.

Schoof H. (2000). The stem cell population of *Arabidopsis* shoot meristems is maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes. *Cell*. **100**: 635-644.

Searle IR, Men AE, Laniya TS, Buzas DM, Iturbe-Ormaetxe I, Carroll BJ, and Gresshoff PM. (2003). Long-distance signaling in nodulation directed by a *CLAVATA1*-like receptor kinase. *Science*. **299**: 109-112.

Smyth DR, Bowman JL, Meyerowitz EM. (1990). Early flower development in *Arabidopsis*. *Plant Cell*. **2**: 755-67.

Stam M, De Bruin R, van Blokland R, VanDer Hoorn RAL, Mol JNM, Kooter JM. (2000). Distinct features of post-transcriptional gene silencing by antisense transgenes in single copy and inverted T-DNA repeat loci. *Plant Journal*. **21**: 27-42.

Stone JM, Trotochaud, AE, Walker JC and Clark SE. (1998). Control of meristem development by *CLAVATA1* receptor kinase and kinase-associated protein phosphatase interactions. *Plant Physiol*. **117**:1217-1235.

THE ARABIDOPSIS GENOME INITIATIVE. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*. **408**: 796 – 815.

Thakur A. (2003). RNA interference revolution. *Molecular Biology and Genetics*. **6**: 1-13.

Trotochaud AE, Hao T, Guang W, Yang Z and Clark SE. (1999). The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and Rho-related protein. *Plant Cell*. **11**: 393-405.

Trotochaud AE, Jeong S and Clark SE. (2000). CLAVATA3, a multimeric ligand for CLAVATA1 receptor-kinase. *Science*. **289**:613-617.

Valvekens D, Van Montagu M, Van Lijsebettens M. (1988). *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc Natl Acad Sci USA*. **85**: 5536-5540.

Weigel D and Glazebrook J. (2002). *ARABIDOPSIS: A Laboratory Manual*. Cold Spring Harbor Laboratory Press.

Wei T, Luo XY, Sanmuels. (2001). Gene silencing: Double stranded RNA mediated mRNA degradation and gene inactivation. *Cell Research*. **11**: 181-186.

Wheeler EA, Baas P, Gasson PE. (1989). IAWA list of Microscopic features for hardwood identification. *International Association of Wood Anatomists*, Leiden, The Netherlands.

Williams RW, Wilson JM and Meyerowitz EM. (1997). A possible role for kinase-associated protein phosphatase in the *Arabidopsis* CLAVATA1 signaling pathway. *Proc. Natl. Acad. Sci. USA*. **94**: 10467-10472.

Winge P, Brembu T, Kristensen R and Bones AM. (2000). Genetic structure and evolution of RAC-GTPases in *Arabidopsis thaliana*. *Genetics*. **156**: 1959-1971.

Ye GN, Stone D, Pang SZ, Creely W, Gonzalez K, Hinchey M. (1999). *Arabidopsis* ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. *Plant J*. **19**: 249-257.

Ye ZH, Droste DL. (1996). Isolation and characterization of cDNA encoding xylogenesis-associated and wounding-induced ribonucleases in *Zinnia elegans*. *Plant Mol. Biol*. **30**: 697-709.

Yin, JQ, and Wan Y. (2002). RNA-mediated gene regulation system: Now and future (Review). *International Journal of Molecular Medicine*. **10**: 355-365.

Yu LP, Simon EJ, Trotochaud AE and Clark SE. (2000). POLTERGEIST functions to regulate meristem development downstream of the CLAVATA loci. *Development*. **127**:1661-1670.

Zhao C, Johnson BJ, Kositsup B, Beer EP. (2000). Exploring secondary growth in *Arabidopsis*. Construction of xylem and bark cDNA libraries and cloning of three xylem endopeptidases. *Plant Physiol*. **123**: 1185-1196.