ABSTRACT

Title of dissertation: *LARSON* DIRECTLY REPRESSES *AGAMOUS* DURING EARLY FLOWER ORGANOGENESIS IN *ARABIDOPSIS THALIANA*

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How cells in a multicellular organism assume their developmental fates and form distinct patterns is a fundamental biological question. To address this question, I studied genetic and molecular regulation of *Arabidopsis* flower organ formation and identity determination. Specifically, how the expression of floral meristem and floral organ identity gene *AGAMOUS (AG)* was regionalized during flower organogenesis. A novel *AG* repressor *LARSON* (*LSN*) was previously isolated in a genetic screen. *lsn* loss-of-function mutations caused precocious expression of *AG* in the inflorescence meristem and ectopic expression of *AG* in sepal primordia, resulting in partial homeotic transfomation of late inflorescences into floral meristems and strong homeotic transformation of first whorl sepals into carpels.

LSN encoded a homeodomain protein that directly bond to *AG cis*-regulatory elements *in vitro*. The *cis*-regulatory elements were conserved in 17 Brassicaceae

species. *LSN* was expressed in a subset of cells located in the peripheral zones of inflorescence and floral meristems. *LSN* expression was significantly reduced in the sepal and petal primordia in wild-type flowers, indicating that repression of *AG* in the sepals and petals was independent of *LSN* transcription. *LSN* might establish epigenetic *AG* repression in the ancestral cells in the peripheral zone to specify the identities of descendant cell types in the floral organs. Therefore, floral organ identities were not only dependent upon gene expression in the organs, but were also dependent upon the histories of the cell development.

Genetic and molecular analyses showed that *LSN* acted upstream of a putative repression complex, which, I proposed, was involved in the maintenance of *AG* repression in flowers. The putative repressive complex consistes of *APETALA1* (*AP1*), *LUNIG (LUG)* and *SEUSS (SEU)* known to encode flower specific repressors of *AG*. Mutations in these three genes enhanced the *lsn* phenotypes. However, none of their proteins interacted with LSN in yeast. Instead, AP1, SEU, and LUG might form a protein complex. Genetic and molecular analyses suggested that the *AG*-repressive functions of the putative complex depended upon *LSN* activity in the peripheral zone of floral meristem. The *AG*-repressive function of *LSN* in the inflorescence meristem was independent of AP1/SEU/LUG putative complex.

LARSON DIRECTLY REPRESSES *AGAMOUS* DURING EARLY FLOWER ORGANOGENESIS IN *ARABIDOPSIS THALIANA*

By

Xiaozhong Bao

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
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Advisory committee:

Professor Zhongchi Liu, Chair
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Professor Stephen Mount
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Professor Heven Sze

Dedication

As this dissertation was about to be written, I realized how much I'm actually interested in life. I'm amazed by the way a cell memorizes its life history and reach out to its destiny.

As this dissertation is about to be written, some fragments of words frequently jump out of my mind. Some are said by a thin old man. What's his name? I don't remember. All I remember is that his lecture was so boring. So boring that I remember clearly that he was pointing to the boring posters hanging on the boring wall with his boring stick when he talked about how a cell become an embryo

Some are said by a bald man, a rare species in China. He liked to joke about everything. "It may be one in a thousand to be bald. It may be one in a thousand to be a professor. You are luck to see a bald professor because I'm one out of a million." I remember he told me: "Everything is in you, genetically. It's never about what the others give to you. It's all about what you can take."

Some are said by a man I called him "the bamboo" because he is tall and I had never saw him bend his body, never. I remember he knew how bone marrow cells changes and changes and finally become lymphocytes. He talked a lot about nonhomologous gene rearrangement, which I once called the anti-bald-y theory because it was so different from what the bald man taught me.

And that lady who promised a treat if I could show her when the big old tree down the hill grew more than a hundred leaves. It didn't that year. It had never grown more than a hundred leaves, so I learnt. She showed me how to grew a plant out of a small piece of a leaf.

But what are their names? Time has passed so quickly. So quick that I did not had a moment to memorize their names. So quick that the fragments of words are still in my mind.

… …

I dedicate this dissertation to those who had said the fragments of words on the campus of Wuhan University.

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To Dr. Jashua Levin, Dr. Steven Hutchisom, and Dr. Robert Martienssen for generously providing *lsn* mutant seeds. Dr. Detlef Weigel for providing KB9 seeds.

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ABBREVIATIONS

acf:	apical carpelloid flower
AG:	*AGAMOUS*
AGI:	Arabidopsis genome initiative
AGO4:	*ARGONUTE 4*
AP1:	*APETALA1*
AP3:	*APETALA3*
ATX1:	*ARABIDOPSIS TRITHORAX1*
bp:	base pair
ca:	carpels
CAL:	*CAULIFLOWER*
CAPS:	cleaved amplified polymorphic sequences
CLF:	*CURLY LEAF*
CLV:	*CLAVATA*
Col:	Columbia
CUC:	*CUPSHAPED COTYLEDON1*
CZ:	central zone
DD:	dimerization domain
DDM:	*DECREASED DNA METHYLATION 1*
DTT:	dithiothreitol
DEF:	*DEFICIENCENS*
EMS:	ethyl methanesulphonate
EMSA:	electrophoretic mobility shift assay
EREBP:	ethylene-responsive element binding proteins
FIL:	*FILAMENTOUS FLOWER*
FLC:	*FLOWER LOCUS C*
FM:	floral meristem
FT:	*FLOWERING LOCUS T*
GA:	gibberelin
Gro:	*Groucho*
GroTLE:	Groucho and transducin-like enhancer family proteins
GUS:	-glucuronidase
H:	histone
HAT:	histone acetyltransferases
HD:	homeodomain
HDAC:	histone deacetylases
IM:	inflorescence meristem
IPTG :	Isopropylthio--D-galactoside
kb:	Kilo-base pair
KN1:	*KNOTTED1*
KNOX:	KNOTTED type homeodomain proteins
LD:	long-day
Ldb:	LIM-domain-binding proteins
Ler:	Landsberg erecta

LFY:	*LEAFY*
LSN:	*LARSON*
LUG:	*LEUNIG*
MADS:	MCM, AG, DEF, and AG type proteins
MCM:	*MINICHROMOSOME MAINTENANCE*
MET:	*METHYLTRANSFERASE*
n:	number
PcG:	polycomb group
PCR:	polymerase chain reaction
pe:	petals
PHD:	plant homeodomain
PI:	*PISTILLATA*
PZ:	peripheral zone
Q-rich:	glutamine-rich
RZ:	rib zone or rib meristem
SAM:	shoot apical meristem
SD:	short-day
se:	eepals
SEM:	scanning electron microscopy
SEP:	*SEPETALA*
SET:	SU(var)3-9, E(Z), and trithorax
SEU:	*SEUSS*
SP:	sepal primordium
SRF:	*serum response factor*
SSLP:	simple sequence length polymorphism
st:	stamens
STM;	*SHOOTMERISTEMLESS*
SUP:	*SUPERMAN*
TALE:	Three amino acid loop extension (A supperclass of homeodomain proteins including IRO, PBC, CUP, TGIF, MEIS, M-ATYP, and BEL classes).
tcf:	terminal carpelloid flowers
TFL1:	*TERMINAL FLOWER1*
TILLING:	Targeting Induced Local Lesions IN Genomes
TrxG:	trithorax group
UFO:	*UNUSUAL FLORAL ORGANS*
W:	whorl
WUS:	*WUSCHEL*
X-Gluc:	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt

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Chapter one

LITERATURE REVIEW

OVERVIEW

How cells in multicellular organisms assume their developmental fates and form distinctive body part patterns are fundamental biological questions. Plants provide excellent examples to systematically unravel molecular elements and cellular processes that govern such complex phenomina. Unlike animals, whose body parts are largely formed during embryonic development, the body parts of sporophytes are formed postembryonically in higher plants. Postembryonic development of plant body parts provides visual and viable phenotypes in mutants that effect the cell fates and body part patterning. The repeatitive productions of physiologically, functgionally, and morphologically identical organs (such as leaves or flowers), essentially if not completely, make plants convenient experimental units for sampling and quantifying phenotypes.

Determination of cell developmental stages and cell identities are largely attribute to the gene expression profiles in the cells. Gene expressions are the downstream effectors of intrinsic and extrinsic signals. The complex multicellularity has evolved twice (three times if you include fungi). Plants provide excellent comparisons with animals for the type of proteins and transcriptional regulatory networks that control cell fates and the body part formations. Plants do not only possess

transcriptional regulators similar to their counterparts in animals, but also contain unique classes of receptors, transcription factors, and other regulatory components.

This review does not intend to examine all the aspects of body parts formation and transcriptional regulations, but provides background information for the genetic and molecular mechanisms that relevant to meristem identities and floral organ identities. The later part of the review discusses the recent advances in the field of eukaryotic transcriptional regulation that are important for formation of a hypothesis about genetic and molecular controls during flower organogenesis for the future tests.

THE MODEL SYSTEM OF PLANT BIOLOGY

Arabidopsis thaliana is a small plant in the mustard family Brassicaceae that has become the model system for studying plant biology and for addressing questions fundamental to all eukaryotes (Meinke *et al.* 1998). More than two decades ago, plant biologists realized the importance of establishing a model plant system, to which community resources, limited funding, and non-duplicated efforts could be devoted. The knowledge and the technologies developed from the studying model system will be, in turn, applicable to most, if not all, other plants and other eukaryotes. The advantageous features of *Arabidopsis*, a simple angiosperm, were then appreciated by a growing number of plant biologists. The modern era of *Arabidopsis* research began in 1987 with opening of the Third International *Arabidopsis* Conference at Michigan State University, and by launching The *Arabidopsis* Genome Initiative (AGI) project in 1996.

Arabidopsis is a small annual weed that naturally occurs throughout Europe, Asia, and North America. Many different ecotypes (or accessions) have been collected from natural populations for experimental analyses. These ecotypes provide excellent material for ecological studies due to their variable genetic backgrounds. The plant can be germinated and grown in media plates, small soil pots, or hydroponic containers. Mature plants reach 15 to 20 cm in height. These features make *Arabidopsis* easy to manipulate in limited laboratory spaces, which is particularly beneficial for genetic screening of large populations. Seeds can be stored without culturing over years in small tubes or bags. The short life cycle (from seed to seed can be completed in ~6 weeks) and the large amount of siblings (5,000 to 10,000 seeds per plant) are especially useful to geneticists who must screen for mutations.

Arabidopsis is a self-fertilizing plant that can be manually out-crossed to produce hybrids. The diploid stage of the plant cell has only 10 chromosomes (n=5). The genome contains 125 megabases of total DNA sequences encoding 25,498 genes, as predicted by the genome sequence (AGI 2000). Mutations can be induced by biological, physical, or chemical agents at the various stages of the plant life cycle. Genes can be introduced easily into the plant genome to establish stable transgenic lines via Agrobacterium-mediated gene transformation. To date, databases and seed banks with collections of wide ranges of sequences, mutants, or transgenic plants are available for public accesses for research, education, and industrial applications. These genetic features and research advances have made *Arabidopsis* a "super model" for molecular

genetic studies, along with *Escherichia coli*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens*.

THE LIFE CYCLE OF ARABIDOPSIS

The life cycle of Angiosperms, such as *Arabidopsis thaliana,* consists of three major stages: endosperm, sporophyte, and gametophytes (Fig 1-1) (Goldberg 1988). The triploid endosperm is developed from the fertilized binucleated central cell in the ovule. Mitosis of the central cell produces a mass of cells (the endosperm) to provide nutrition to the growing embryo. The sporophyte, the dominant stage of a flowering

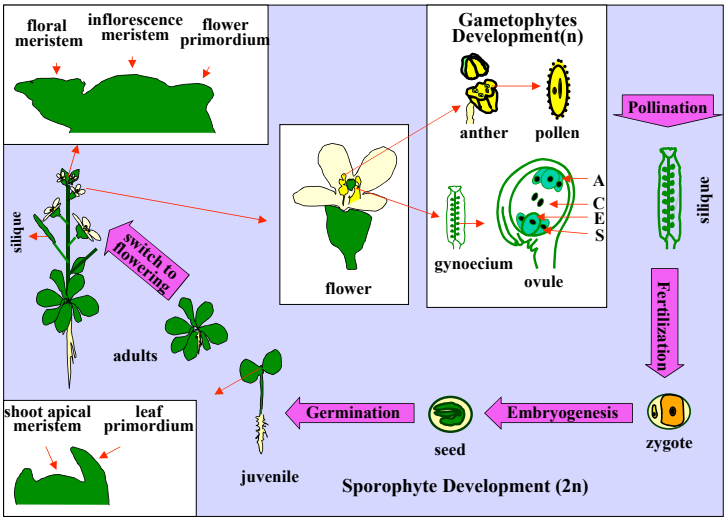


Fig 1-1. A schematic presentation of *Arabidopsis thaliana* life cycle. Like other flowering plant, the gametophytes development happens in the anther or ovary of the sporophyte. The sporophyte development consists of three distinguishable phases: seed, juvenile plant, and the adult plant. The above-ground parts of plant body are developed or derived from the shoot apical meristem. A, antipodals; C, central cell; E, egg cell; S, synergids.

plant, starts from a fertilized egg, a zygote (2n). The fertilized egg develops into the embryo in the gynoecium (ovary) of the parental plant. Embryogenesis produce a dormant seed, in which lay the endosperm-surrounded embryo. Seeds mature within the slender fruits known as the siliques. Under suitable environmental conditions (such as light, temperature, and humidity), the dormant seed germinates and produces new parts or organs that are not present in the embryo. Groups of pluripotent cells, called meristem cells, the equivalent of somatic stem cells in animals, are located at the two ends of the mature embryo. Self-renewable meristem cells are responsible for supplying undifferentiated cells for organ formation during postembryonic development. Under suitable environmental conditions, seeds germinate into seedlings (juvenile plants) and then adult plants, which develop through vegetative and reproductive phases. The reproductive organs of an *Arabidopsis* flower include six stamens (male) and a gynocium formed by two fused carpels (female). The outgrowth of sub-epidermal cells on the adaxial wall of ovary forms ovules, arranged in four rows along the placental region. The megaspore mother cell inside the ovule undergoes meiosis and produces 4 haploid megaspores. As three of these megaspores degerate, one of them undergos three mitotic divisions and differentiate into an egg cell surrounded by two synergids, a central binucleat cell, and three antipodals (Reiser & Fischer 1993). Each microspore mother cell in the anther of a stamen undergoes meiosis to form four microspores. Mitosis of microspores produces pollen grains, each of which contains a vegetative cell and a generative cell. The generative cell divides one more time to produce two sperm cells during pollen germination. One of the sperm

will fertilize the egg to form the zygote and the other one will fertilize the binucleat central cell to further develop into the triploid endosperm (McCormick 1993).

MERISTEM ARCHITECTURE AND ORGANIZATION

Meristem architecture

Like all other angiosperms, post-embryonic development of *Arabidopsis* gives rise to the dominant body parts of a plant. This development starts from pluripotent meristem cells that are localized in regions such as the shoot apex and the root tip. The shoot apical meristem (SAM) gives rises to leaves and shoots during the vegetative phase and flowers during the reproductive phase. The ancestral cells that are destined to form leaf or flower primordia are called initials. The positions of the initials in the meristem are reflected by phyllotaxy, the subsequent arrangement of derivative structures around the stem, along the centripetal axis. *Arabidopsis* has a spiral phyllotaxy with a defined angle of 137.5^{o} between two subsequent organs. A node forms when a primordium is initiated on the meristem. The stem separating two nodes is named internode. In vegetative plants, the meristem gives rise exclusively to leaf perimodia with extremely short internodes. When *Arabidopsis* plants switch to reproductive growth, flowers are produced instead of leaves, accompanied with rapid internode elongation (bolting). The reproductive SAM is then called an inflorescence meristem (IM) to distinguish it from a vegetative SAM. The IM gives rise to floral primordia exclusively. Floral primordia maintain the self-renewing ability and are called floral metistems (FM) due to its ability to produce a set of floral organs. As the primary shoot bolts, apical dominance decreases, allowing development of axillary

meristems in the axils of rosette leaves and cauline leaves (Irish & Sussex 1992, Hempel & Feldman 1994). Axillary meristems possess developmental potentials similar to those of the primary shoots.

Meristem cell organization

The *Arabidopsis* inflorescence and floral meristems have a tunica-corpus duplex structure with three distinctive cell layers (Sussex 1989). In *Arabidopsis*, the outer L1 and L2 layer cells comprise the tunica (Fig 1-2) (Irish & Sussex 1992, Leyser & Furner 1992, Medford *et al.* 1992). The L1 cells divide exclusively anticlinal (perpendicular to the surface) to give rises to the epidermis. L2 cells divide anticlinally within the meristem but may divide in other planes during differentiation. L3 cells, which make up the corpus, can divide in all planes. Both L2 and L3 contribute to the body of the plant in proportions that vary in different organs.

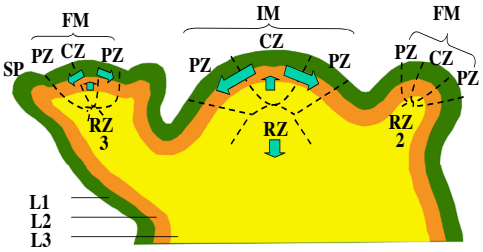


Fig 1-2. A schematic diagram of meristem organization. FM and IM have essentially the same cytohistological zonation pattern superimposed on the tunica-corpus organization. L1 and L2 cell layers make up the tunica and L3 cell layer is the corpus. Numbers indicate the flower stages. Arrows indicate the directions of cell position changes due to cell divisions. CZ, central zone; FM, floral meristem; IM, inflorescence meristem; PZ, peripheral zone; RZ, rib zone or file meristem; SP, sepal primordium.

Arabidopsis SAM (or IM) consists of approximately 60 cells. As in other flowering plants, the cytohistological zonation pattern is superimposed on the tunica-corpus organization (Steeves & Sussex, 1989). The central zone (CZ) at the summit of the apical dome has relatively large and highly vacuolated cells that divide relatively slower than the surrounding cells. These cells provide undifferentiated cells to the rest of the regions of meristem. Underneath the central zone, a group of cells called the rib zone (RZ) or file meristem zone gives rises to the internal tissues of the stem. The central zone is surrounded by a doughnut-shaped region called the peripheral zone (PZ) that consists of smaller cells with inconspicuous vacuoles. The peripheral zone contains initial cells that will divide and form the primordia of derivative structures afterwards along the centripetal axis.

The organization of FM is similar to that of the IM. FM consist of the duplex tunica-corpus cell layers and CZ provides progeny cells to initiate organogenesis in the PZ (Steeves & Sussex 1989). Genetic studies support the view that flowers are modified shoots and floral organs are modified leaves (Bowman *et al.* 1989, Honma & Goto 2001, Weigel *et al.* 1992). Most genes that are involved in the maintenance of the IM, also participate in FM organization and maintenance.

Genetic regulation of meristem establishment and maintenance

By studying phenotypes of mutant plants that lack meristem cells or that accumulate ectopic meristem cells, the genetic mechanisms underlying the regulation of meristem cell maintenance and development are being revealed. The *Arabidopsis* gene

SHOOTMERISTEMLESS (*STM*) is required for establishing an embryonic SAM (Barton & Poethig 1993). Seedlings of loss-of-function *stm* mutants germinate with roots, hypocotyls, and cotyledons, but without a SAM. *STM* encodes a homeodomain protein, a homolog of *KNOTTED1* (*KN1*) from maize. *STM* mRNA is found earliest in one or two cells of the late globular stage embryo and subsequently spreads to form a stripe between the presumptive cotyledon primordia. *STM* expression is reduced at the emerging lateral organ primordia. These observations suggest that first, STM inhibits cell differentiation and organ outgrowth, and second, genes that define organ identities may repress *STM* expression in the differentiated organs. Along with *CUPSHAPED COTYLEDON1* (*CUC1*) and *CUPSHAPED COTYLEDON2* (*CUC2*), whose expression patterns overlap with that of *STM* in early stages of embryo development, *STM* is proposed to have a role in separating organs initiated from the SAM (Aida *et al.* 1997). Plants that have these genes mutated develop fused organs.

STM is active in the SAM and FM and is required for meristem maintenance, replenishing undifferentiated cells to the central zone. Maintenance of the meristem also requires another important homeobox gene, *WUSCHEL* (*WUS*). *WUS* expression is established in sub-apical cells of 16-cell stage embryos and becomes restricted to a small number of cells in the bottom of the meristem central zone (Mayer *et al.* 1998). Two lines of evidence show that *WUS* is not required for meristem initiation. First, *STM* is expressed in *wus*, although in fewer cells than in wild-type plants. Second, axillary inflorescences or abnormal flowers are still produced by *wus* mutant plants. *wus* plants initiate defective shoot meristems repetitively, which then terminate

prematurely in aberrant flat structures. Thus, *WUS* is important for meristem integrity and maintenance (Laux *et al.* 1996).

While loss-of-function mutants of *STM* and *WUS* result in a reduction or loss of stem cells, loss-of-function mutants of *CLAVATA* genes (*CLV1*, *2*, and *3*) result in enlarged or fascinated SAM and FM (Clark *et al.* 1993, Clark *et al.* 1995, Kayes & Clark 1998). Confocal laser scanning microscopy revealed that the SAM of *clv* mutants contains more cells than that of wild-type plants. These observations indicate that the function of *CLV1, 2,* and *3* is to restrict the number of meristem cells. However, the mitotic index of stem cells in the *clv* meristem central zone is not higher than that of wild-type plants. Therefore, the maintenance of cell population size at the central zone is achieved by controlling the cell maturation rate, not by controlling the mitotic cell division rate. In other words, *clv* mutants result in the accumulation of pluripotent stem cells at central zone by slowing down the rate by which the central zone cells differentiate into the peripheral zone cells to form organ primordia. Consistent with this interpretation, floral organ number is increased in *clv*, indicating extra cells are allocated into additional floral organ primordia.

CLV1 encodes a monospan transmembrane protein with extracellular leucine-rich repeats and an intracellular serine/threonine kinase domain (Clark *et al.* 1997). CLV2 is a receptor-like protein consisted of extracellular leucin-rich repeats, one transmembrane domain, and a short cytoplasmic tail (Jeong *et al.* 1999). The *CLV3* gene, however, encodes a 96-amino acid extracellular protein (Fletcher *et al.* 1999, Rojo

et al. 2002). Expression patterns of these genes provided important clues to how they control the size of meristem cell population. *CLV1* and *CLV3* mRNA are observed during embryogenesis starting at heart stage (Clark *et al.* 1997, Fletcher *et al.* 1999). After germination, *CLV2* mRNA can be detected in all shoot tissues (Jeong *et al.* 1999). *CLV3* mRNA is observed in the L1 and L2 cells of the central zone and *CLV1* mRNA is restricted in the L3 layer cells in both IM and FM (Clark *et al.* 1997, Fletcher *et al.* 1999). Genetic and molecular studies suggest that CLV3 acts as the ligand for CLV1 receptor kinase, which heterodimerizes with CLV2 on the plasma membrane and mediates signaling pathways to promote cell differentiation (Clark 2001).

stm and *clv* mutually suppress each other in a dominant fashion, indicating that the *clv* phenotype is sensitive to the level of *STM* activity. Thus, *STM* and *CLV* loci may act in parallel pathways to competitively regulate SAM function (Clark *et al.* 1996, Kaya *et al.* 1998). *WUS*, however, seems to act downstream of *CLV* signal because *wus* mutant phenotype is epistatic to *clv* phenotype at the SAM (Brand *et al.* 2000, Schoof *et al.* 2000). *WUS* is expressed in apical cells prior to the initial appearance of *CLV1* and *3* during embryogenesis. As the plant matures, *WUS* mRNA is restricted to a small group of cells at the bottom of the shoot apical and floral meristems beneath the *CLV3* expression domain and partially overlaps with the *CLV1* domain in the deeper L3 cells. The *WUS* expression domain is expanded in *clv1* or *3* mutants, accompanied with enlarged meristems. *CLV3* over-expression abolishes *WUS* mRNA, causing meristem arrest. Therefore, the *WUS* and *CLV* signaling pathway forms a regulatory feedback loop to determine the sizes of cell populations in the SAM and the FM.

Homeobox Genes

Walter J. Gehring and his colleagues published two studies in 1984 reporting a evolutionarily highly conserved protein domain, homeodomain (HD), in genes whose mutants exhibited homeotic transformations in animals (McGinnis *et al.* 1984a, McGinnis *et al.* 1984b). The *Nature* paper mis-printed the name to "homeobox" and this term has been preserved ever since. In the past twenty years, a large number of homeobox genes or putative homeobox genes have been discovered in genomes of eukaryotes across species. These genes share a common 180 bp DNA sequence encoding a 60-amino acid HD consisting of three α-helices separated by a loop and a turn (Fig 1-3). The most conserved is the third helix, resting in the major groove of the DNA double helices and serving as the recognition helix (Gehring 1994). Homeobox proteins primarily serve as transcription factors that regulate target genes in precise spatial and temporal patterns. This transcriptional regulation controls cell fate specificity, body plan, and provides positional information along the anterior-posterior axis in animals. A variety of missense mutations have been described affecting cell fate specification and development of fungi, plants, and animals (Gehring 1994, Hughes & Kaufman 2002, Patterson & Potter 2003). In humans, missense mutations in homeodomains often lead to structural abnormalities, organ malfunctions, or cancers (Fig 1-3) (D'Elia *et al.* 2001).

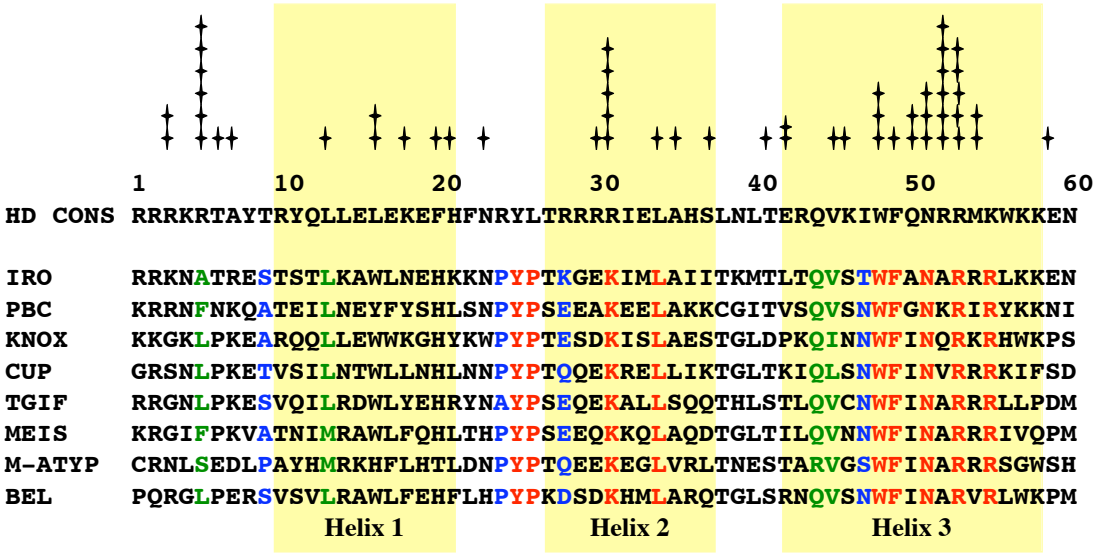


Fig 1-3. Homeodomain concensus and missense mutations in human. The homeodomain proteins play important roles in cell and developmental biology of all eukaryotic organisms. Missense mutations (frequencies and positions are indicated by ✦) give rise to multiple severe human diseases (D'Elia *et al.* 2001). Homeodomain consensus (HD CONS) and members of TALE (three amino acid loop extension) superclass are shown (Bürglin 1997, Gehring *et al.* 1994). Conserved amino acid residues in the superclass are colored (red, identical; blue, highly conserved; green, similar). Numbers indicate positions in HD.

The first plant homeobox gene *KNOTTED1* (*KN1*) was isolated from a dominant maize mutant that altered leaf development (Vollbrecht et al 1991). Multiple homeobox gene families have been found in plants since then, based on sequence homology to

KN1 or mutagenesis screens (Chan *et al.* 1998). Nearly one hundred (98) homeobox genes have been predicted by the completion of *Arabidopsis* genome sequencing, including 17 homeodomain-bZIP that is not found in *Drosophila* or yeast (AGI 2000).

Conservation of the homeodomain suggests that ancestral homeobox genes may have existed in the putative unicellular organism that gave rise to the lineages leading to animals, plants, and fungi. Although the secondary structures of homeodomains are highly conserved in each superclass, plant homeobox gene families often bear unique structures on the N-terminal arms differentiating themselves from their homologs in animal or other systems (Bürglin 1997, Bürglin & Cassata 2002, Chan *et al.* 1998, Gehring *et al.* 1994). Interestingly, the plant KNOX class, represented by *KN1*, share a conserved domain upstream of the homeodomain with animal MEIS and PBC classes (Bürglin 1998). These three classes, thus, may have coevolved before the divergence between plants and animals. Similarly, PHD (plant homeodomain) proteins, formerly thought to be specific to plants, are now found in human and mouse (Aasland *et al.*, 1995).

Homeobox genes have diverse functions in plants. KNOX class genes such as *STM* and *KN1* are important for the body plan and organ positioning along the plant centripetal axis (Hake & Char 1997, Long *et al.* 1996). The homeodomain-bZIP family controls transcriptional responses to light, hormones, and stresses (Schena and Davis 1992). The PHD family bears a cysteine-rich region N-terminal to the homeodomain that is is thought to be important for protein interactions, redox sensitivity, and DNA-

binding specificity (Schindler *et al.* 1993, Uberlacker et al 1996). Glabra family, with a long N-terminal arm carrying a dimerization domain, seems to be involved in fate determination of specialized epidermal cells such as trichomes and root hairs (Rerie et al 1994, Masucci *et al.* 1996). *WUS* probably represents a novel homeobox gene family unique to plants, functioning in meristem cell development. *BELL1* is the prototype of the BEL class (discussed in chapter three) that is unique to plants (Reiser *et al.* 1995).

FLOWERING

Phase transitions

Beside the genes required for meristem establishment and maintenance, meristem identity genes are required to define the type of lateral organs produced from a meristem. SAM is formed in embryogenesis but its function as the source of organ production is only realized after the juvenile phase. After germination, the interactions between environmental cues and the autonomous developmental program determine the activities of meristem identity gene and thus set the planta to a phase transition from the adult vegetative phase to the reproductive phase. The SAM functions change from producing exclusively leaves to producing floral meristems, which in turn produce floral organs. Thus, the most drastic and significant event of a plant's life cycle, changes in organogenesis, occurs through the phase transitions at the meristem via changes in the activities of meristem identity genes. The timing of the vegetative to reproductive phase transition (or flowering) can be measured by the number of rosette leaves produced before the production of the first flower primordium.

Three major theories have been postulated to explain the mechanisms underlying flower induction. The "florigen/antiflorigen" hypothesis proposes that floral promoter and inhibitor are each specific biochemical factors (Lang 1984). The "nutrient diversion" hypothesis suggested that floral induction is a means of modifying the source/sink relationships within the plant in such a way that the shoot apex receives a better supply of assimilates in inductive conditions than noninductive conditions (Sachs and Hackett 1983). The "multifactorial control" theory postulates that several assimilates and phytohormones participate cooperatively in floral induction (Bernier *et al.* 1981). Genetic analyses have revealed, so far, five epistatical classes of pathways involved in floral induction: autonomous, vernalization, gibberelin (GAs), photoperiod, and thermal clock (Fig 1-4) (Mouradov *et al.* 2002, Poethig 2003, Simpson *et al.* 1999, Simpson & Dean 2000, Soltis *et al.* 2002). Signals from these pathways may be integrated at the cellular and molecular levels to activate meristem-identity genes (Miguel & Weigel, 2000).

Autonomous pathway

Arabidopsis is an annual flowering plant that shows a facultative (or quantitative) response to environmental cues such as temperature and photoperiod. However, the environmental cues can only affect the plants that are competent to respond. The autonomous pathway is essential to prepare the plants to a state of readiness for flowering inductive cues. The autonomous pathway is speculated to monitor the developmental age of a plant; the plant must pass through a juvenile phase before it will flower. Mutations in this pathway cause late flowering in both long-day

(LD) and short-day (SD) conditions (Koornneef *et al.* 1998). These genes promote flowering independent of environmental signals, and thus, they probably monitor or respond to endogenous cues such as an internal developmental clock.

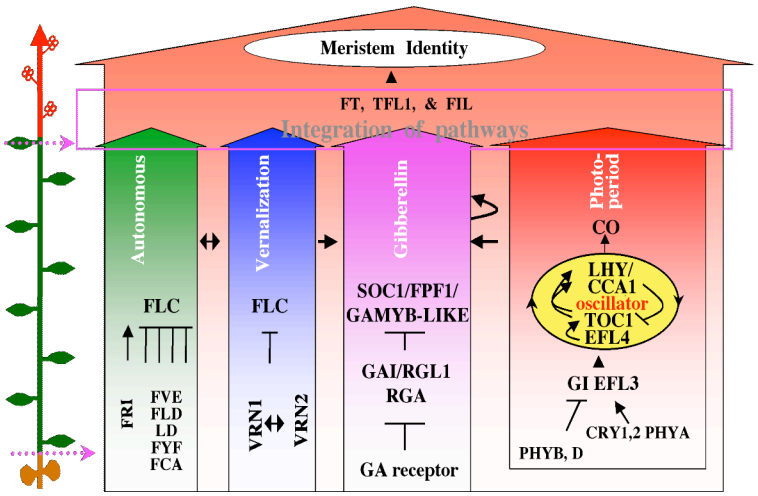


Fig 1-4. Five genetic pathways cooperatively induce flowering in *Arabidopsis*. Accumulation of thermal time (growth degree-days, the orange back ground) may incorporate and synchronize the autonomous, vernalization, gibberelin, and photoperiod pathways to promote flowering. Plant developmental stages are indicated by the schematics of a plant on the left. Major genes and their regulatory relationships are indicated for each pathway (vertical arrows).

Vernalization pathway

Vernalization, a cold temperature treatment typifying a winter season in temperate latitudes, promotes flowering in *Arabidopsis,* but is not essential. Vernalization is a slow and quantitative process in which increasing periods of low temperature cause progressively earlier flowering until a saturation point is reached. Different ecotypes of *Arabidopsis* show tremendous natural variation in the extent to

which their time requirement for flowering is shortened by vernalization. This variation had led to genetic analyses of the vernalization pathway (Napp-Zinn 1985). The vernalization pathway shares a number of genes with the autonomous pathway, and cold treatments can compensate for a lack of autonomous pathway genes.

Perception and response to vernalization are separated temporally. Vernalization prepares the plant to flower, but does not evoke flowering, which occurs only after an extended period of growth at warmer temperatures. The perception of and response to low temperature is localized in the meristem cells of embryos and seedlings. However, the response (flowering) does not occur immediately upon being raised to higher temperature, but weeks later untill signals from other pathways have been integrated. The "vernalized state", thus, has to be maintained through many mitotic divisions epigenetically (Finnegan *et al.* 1998, Sheldon *et al.* 2000).

Gibberellin pathway

Gibberellins, constituting a large family of diterpene acids, have an important role in promoting flowering in *Arabidopsis*. GAs are synthesized in shoots, actively growing buds, leaves, and upper internodes. A high level of GA promotes multiple processes related to flowering such as primordium initiation and bolting. Blocking GA synthesis abolishes flowering in short SD, but has minor effects in LD (Wilson *et al.* 1992). The gibberellin pathway interacts readily with the vernalization pathway, the photoperiod pathway, and the other physiological or signal transduction pathways that

are involved in plant development and/or responses to environmental conditions (Olszewski *et al.* 2002).

Photoperiod pathway

Arabidopsis is a quantitative long day plant, in which flowering can eventually occur in SD and is accelerated by LD. As in other flowering plants, *Arabidopsis* monitors day length by measuring the length of the night, or the timing of a night break, via sensing red/far-red light ratio at dawn and blue-light at dusk using photoreceptors. Three physiological models of photoperiodic time measurement have been proposed, as reviewed by Yanovsky and Kay (2003). Genetic and molecular data support the external coincidence model (Bünning 1960): that circadian clock drives a rhythm in a light-sensitive process, and that photoperiodic responses are observed when the illuminated part of the day overlaps with the inducible phase of the endogenous circadian rhythm. Leaves are largely responsible for photoperiod perception. Leaf grafting and root girdling experiments have shown that the chemical signals from leaves are transmitted via phloem, passing through the root, to the SAM (Bernier 1993, Tooke & Battey 2000).

Thermal clock pathway

Thermal time (or growing degree-days) is defined as the accumulation of temperature differences (the environmental temperature minus the species-specific basal temperature at which growth ceases) over time (Cao & Moss 1989). Under certain light regimes, the major events of plant life cycle, such as leaf initiation, rate and duration of

leaf growth, and flowering time, are linearly related to thermal time. Genetic and molecular studies provided new evidence supporting thermal time regulation of flowering time (Blazquez *et al.* 2003, Halliday *et al.* 2003). Plants can produce more rosette leaves before flowering at low temperatures (~16 ^{o}C) than at high temperature (~26 ^{o}C). High temperature up-regulates the expression of flowering promotion gene *FLOWERING LOCUS T* (*FT*), which is also regulated by photoperiod. The autonomous pathway genes *FCA* and *FVE* appeared to be essential in temperature responses: mutations in either gene eliminate the temperature-induced differences in flowering time. Based on these data, Poethig (2003) proposed a thermal clock regulatory pathway, by which plants can integrate information from other flowering pathways. Interestingly, flowering time responds to day and night temperatures differently (Thingnaes *et al.* 2003), implying the oscillator entrainment by temperature. This agrees with the observation that light modulates temperature responses via the *PHY B* signaling pathway (Halliday *et al.* 2003), which acts upstream of the oscillator entrainment by the photoperiod.

Although the five pathways are often studied under isolated situations, interactions among them are well recognized in the literature (Mouradov *et al.* 2002). Mutations in one pathway may be compensated by manipulating the environmental conditions or by mutations of the other pathways (Simpson *et al.* 1999, Soltis *et al.* 2002). The coupling among pathways may be crucial for the pathways to maintain a level of synchrony and to be integrated at certain points to activate meristem identity genes.

INFLORESCENCE AND FLORAL MERISTEM IDENTITIES

Determinations of inflorescence meristem and flower primordia initiation are separate events (Huala and Sussex 1993, Okamuro *et al.* 1993, Ratcliffe 1999). Mutations in FM identity genes do not disrupt phase transition but disrupt the formation of normal flowers (Weigel *et al.* 1992). Thus, genes involved in inflorescence and floral meristem identities shall be considered separately, not excluding the possibility that a gene may be involved in both events. Based on this argument, inflorescence meristem identity genes have the following characteristics: first, their own activities and their effector activities are found in the shoot apex (not excluding detection in the other parts of a plant); second, their expression is regulated by flowering inductive or inhibitory factors; and third, the absence of these gene products results in altered flowering time and/or inflorescence architecture. In the other words, inflorescence meristem identity genes are the downstream targets of flowering time regulatory pathways.

Two homologous genes *FT* and *TERMINAL FLOWER 1* (*TFL1*), encoding proteins similar to phosphatidyl ethanolamine binding proteins, act in an antagonistic manner to determine the inflorescence meristem identity (Kardailsky *et al.* 1999, Kobayashi *et al.* 1999). *FT* is regulated by both the autonomous and the photoperiod pathways. Reduced or increased expression of *FT* results in late or early flowering, respectively. Having more than 50% amino acid sequence identity, *TFL1* gene acts nearly in the opposite to *FT*. However, these two genes do not interfere with each

other's expression level or protein functions. Instead, they act, at least partially, in parallel to regulate different downstream genes (Shannon & Meeks-Wagner 1993).

FILAMENTOUS FLOWER (*FIL*) is suggested to be another inflorescence meristem identity gene expressed on the abaxial side of organ primordia (Sawa *et al.* 1999a Sawa et al 1999b). Mutations in *FIL,* which encodes a protein with zinc finger and HMG-related domains, result in a slightly early flowering phenotype.

The definitive functions of inflorescence meristem identity genes are to initiate floral meristem and to control floral meristem cell population. Floral meristem identity genes, whereas, activate floral organ identity genes to produce floral organs. In *Arabidopsis*, the floral meristem is not a continuation of inflorescence meristem, but is initiated from the initial cells located in the peripheral zone of inflorescence meristem. The floral meristem has its own CZ and PZ. *APETALA1 (AP1), AGAMOUS (AG), CAULIFLOWER (CAL), FIL, LEAFY (LFY)* and *UNUSUAL FLORAL ORGANS (UFO)* are essential for initiating and regulating the function of promoting floral organ initiation (Fig 1-5) (Komaki *et al.* 1988, Kunst *et al.* 1989, Irish & Sussex 1990, Bowman *et al.* 1989, Kempin *et al.* 1995 Levin & Meyerowitz 1995, Weigel & Nilsson 1995, Sawa *et al.* 1999a, b). Mutations in these genes, especially when more than one genes are mutated, floral meristems can be converted to shoots or abolished (Chen *et al.* 1999, Liljegren *et al.* 1999, Sawa *et al.* 1999a, b, Okamuro *et al.* 1993). For example, floral meristems are partially converted to determinate shoots with the morphology resembling "green flowers" in *lfy* plants (Huala & Sussex 1992, Weigle *et al.* 1992). In

lfy ap1 double mutants, flowers are replaced by lateral shoots characterized with extended internodes, spiral phyllotaxis, and an indeterminate pattern of growth. *TFL1* is an important spatial regulator of *LFY*, such that *LFY* becomes ectopically expressed in the *tfl1* inflorescence meristem (Weigel *et al.* 1992).

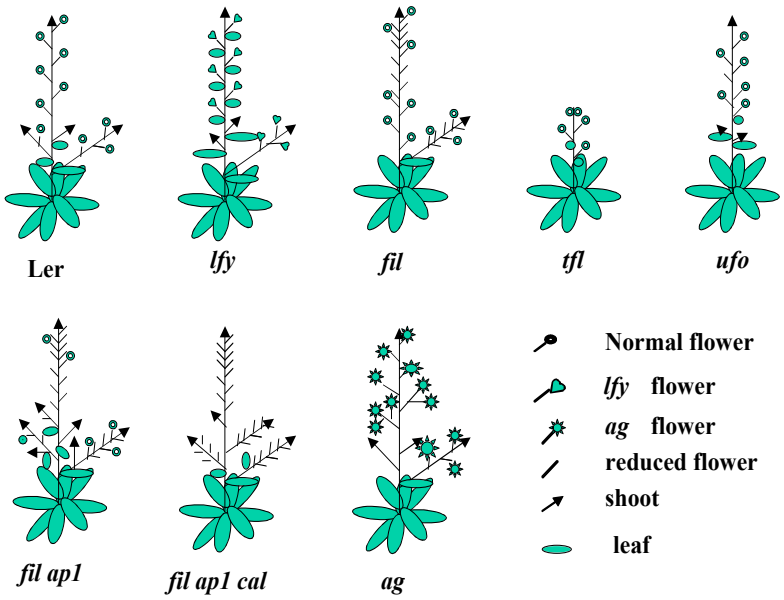


Fig 1-5. Inflorescence meristem identity genes and mutant phenotypes. Mutations in these genes either abolish flowers or produce flowers at wrong time or in wrong places. Inflorescence meristem genes may also be involved in determining floral meristem identity or/and floral organ identity because mutations of these genes may alter floral organ production or morphology.

The floral meristem is terminated after producing a full set of floral organs. In contrast, inflorescence meristem continuously produces flowers on its flanks until the end of the natural age of the plant. *AG* plays an important role in regulating the determinacy of flora meristems (Bowman *et al.* 1989, Lenhard *et al.* 2001, Lohmann *et al.* 2001). A lack of *AG* activity results in the repeated production of floral organs and axillary flowers. This phenotype is due to continuous and elevated *WUS* expression in

the floral meristem of *ag* mutant plants. *WUS* is normally repressed by *AG* at stage 6 flowers to terminate floral meristems in wild-type flowers. The absence of *AG* activity leaves *CLV-WUS* feedback loop unregulated and sustained (Schoof *et al.* 2000).

Some floral meristem identity genes, such as *AP1* and *AG*, are also floral organ identity genes that regulate floral organ identity (see below). Thus, loss-of-function mutants often display abnormal floral organogenesis, producing under-developed, undifferentiated, or homeotically transformed floral organs.

FLORAL ORGAN IDENTITY AND PATTERNING

Floral organ determination and FM determination are separate events (Huala and Sussex 1993, Okamuro *et al.* 1993). Evidently, leaves can be converted to floral organs (stamens or petals) if the appropriate floral organ identity genes are over-expressed, bypassing the need for floral meristems (Pelaz *et al.* 2001, Honma & Goto 2001). Floral organ identities are defined by a small group of genes encoding transcription factors know as the A, B, C, and E class genes.

The ABCE model

Arabidopsis flowers consist of four basic organ types: sepals, petals, stamens, and carpels arranged in four concentric whorls from outside to inside. Despite dramatic variations in the number, color, and shape of floral organs in different species, this arrangement is fixed in the majority of the angiosperm species. This patterning similarity may reflect the similarity of molecular genetic controls for organogenesis.

Isolation of homeotic mutants and genetic analysis using these mutants in *Arabidopsis* and *Antirrhinum majus* led to the proposal of the ABCE model (Bowman *et al.* 1989, Bowman *et al.* 1991, Coen & Meyerowitz 1991, Pelaz *et al.* 2000, Meyerowitz *et al.* 1989). This model suggests that the identities of floral organs are controlled by four classes (A, B, C, and E) of homeotic genes (Fig 1-6). These classes of organ identity genes work in a combinatorial fashion. Each whorl of organs is defined by a unique combination of one to three different classes of organ identity genes. Each class is consisted of one to several genes. A class genes *AP1* and *AP2* specify sepals in whorl 1. Combination of A, B (*APETALA3 (AP3), PISTILLATA (PI)*), and E (*SEPETALA1, 2, 3(SEP1/2/3)*)class genes determines petal identity in whorl 2. B, C (*AG*) , and E class genes together determine stamen identity in whorl 3. Finally, C and E class genes *AG* and *SEP1/2/3* determine carpel identity in whorl 4.

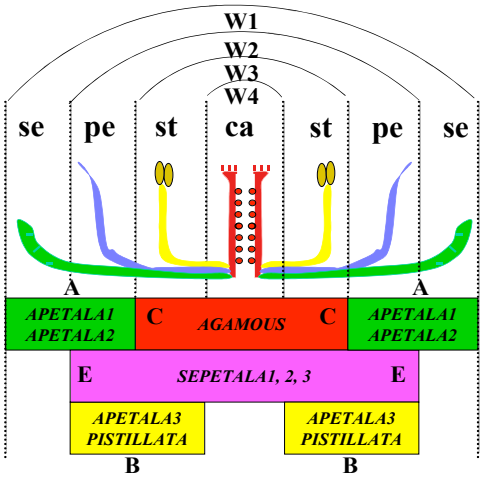


Fig 1-6. Floral organ identities are determined by the ABCE model. Floral organs are arranged in four concentric whorls (W1 to W4). Sepals (se), petals (pe), stamens (st), and carpels (ca) are grown in the outmost W1 to innermost W4, respectively. The combination of two to three classes of homeotic genes that are active in the same whorl determines the organ identity in that whorl. Colored rectangles indicate functional regionalization understood to date.

Mutations in each of A, B, and C classes of genes alter the identity of organs in two adjacent whorls. A complete loss of A (*ap1* or *ap2*) functions causes transformation of the whorl 1 sepals into carpels and whorl 2 petals into stamens. A loss of B (*ap3* or *pi*) function results in sepals instead of petals in whorl 2 and carpels instead of stamens in whorl 3. Finally, abolishing the C (*ag*) activities transforms stamens into petals in whorl 3 and carpels into (sepal-petal-petal)n repeats in whorl 4. Each member in A, B, and C classes is necessary but insufficient for specifying the corresponding organ identity. Different from A, B, and C genes, E class mutations affect the identities of the inner three whorls (Bowman *et al.* 1989, Bowman *et al.* 1991, Coen & Meyerowitz 1991, Meyerowitz *et al.* 1989, Pelaz *et al*. 2000). Sepals are produced in whorl 2, 3, and 4 if and only if all three redundant E class genes *SEP1, 2,* and *3* are mutated (Pelaz *et al.* 2000). Under the notion that floral organs are specialized leaves, sepals are less specialized than any other floral organs. Thus, *SEP1/2/3* gene activities are likely to provide readiness for further specification of floral organs in the inner three whorls of a flower.

Protein multimers may be required to carry out biological functions of floral organ identity genes. Over-expression of only selected combinations of organ identity genes are insufficient to convert vegetative leaves to floral organs (Krizek & Mereyowitz 1996, Honma & Goto 2001, Pelaz *et al.* 2001, Mizukami & Ma 1992). Transgenic plants over-expressing protein combinations from A, B, and E classes (PI/AP3/AP1, PI/AP3/SEP3, AP1/AP3/PI/SEP3, or AP1/AP3/PI/SEP2/SEP3) converted leaves into petals. Whereas over-expression of B, C, and E classes (PI/AP3/SEP3/AG)

converted leaves to stamens. Yeast 2-hybrid assays and in vitro pull-down assays detected not only heterodimers such as AG/SEP1, AG/SEP2, AG/SEP3, and AP1/SEP3, but also heterotrimers such as AP1/AP3/PI and AP3/PI/SEP3 (Goto and Meyerowitz 1994, Honma & Goto 2001, Pelaz *et al.* 2000). It was proposed that multimers are most likely to occurr in floral organs (Egea-Cortines *et al.* 1999, Honma & Goto 2001). Although these data do not prove which protein complexes determine petals or stamens in plants, they clearly suggest that some MADS-box proteins are partially interchangeable.

Homeotic genes

Floral organ identity genes are a special group of homeotic genes (or sector genes) functioning in different flower organs. Homeotic genes encode transcription factors that determine cell fate and organ development in eukaryotic systems. Mutants of homeotic genes cause homeosis: transformation of the body part, where a homeotic gene is normally functioning, to another body part, where the homeotic gene is normally absent. Therefore, all of the eight floral organ identity genes discussed above are homeotic genes.

AP2 is the prototypic member of AP2/EREBP (ethylene-responsive element binding proteins) family of transcription factors (127 members in *Arabidopsis*) that are unique to plants (AGI 2000, Riechmann & Meyerowitz 1998). AP2/EREBP type proteins contain either one or two 70-amino acid domains called AP2 domains that are responsible for DNA binding specificity and affinity (Nole-Wilson & Krizek 2000).

Each of the AP2 domains is folded into a novel DNA recognition structure consisting of a three-stranded β-sheet packed against an α-helix. Residues in the β-sheet are primarily responsible for DNA recognition (Allen *et al.* 1998). The first AP2 domain is proposed to contact the 5' half of the binding site on the DNA template and the second domain contacts the 3' half (Nole-Wilson & Krizek 2000). AP2/EREBP genes play a variety of roles throughout the plant's life cycle, such as floral organ identity determination, control of leaf epidermal cell identity, and responses to various types of biotic and environmental stresses (Riechmann & Meyerowitz 1998).

The rest of the floral organ identity genes (*AP1, AP3, PI, AG,* and *SEP1/2/3*) belong to the ancient MADS-box gene family that has been found in many eukaryotes. The name is taken from the founding members, *MINICHROMOSOMEMAINTENANCE 1* (*MCM1*) in yeast, *AG* in *Arabidopsis*, *DEFICIENCENS* (*DEF*) in *Antirrhinum*, and Serum Response Factor (SRF) in human (Riechmann & Meyerowitz 1997). Plants have, by far, the largest number, evidenced by 56 annotated genes and about 40 of which are molecularly isolated in *Arabidopsis* (AGI 2000, Ng & Yanofsky 2001). MADS-box floral organ identity genes share a 56-amino acid MADS domain at the N-terminus (including an α-helix and a hydrophobic patch), a coiled-coil K domain (66 aa), a weakly conserved I domain linking the MADS domain and K domain, and a divergent C-terminal region. The I and K domains are involved in protein-protein interactions. The C region might function both in transcription activation and in stabilizing protein-protein interactions (Riechmann & Meyerowitz 1997).

An X-ray crystallography study of SRF protein showed that MADS-box proteins dimerize, via antiparallel oriented short α-helices folding over β-sheets in the I domain, when they bind DNA (Pellegrini *et al.* 1995). The primary DNA-binding element is the α-helix of the MADS domain that sits in the minor groove of the target site. The extending amino terminal of the dimmer penetrates into the minor groove of DNA helix, making additional contacts. MADS-box proteins recognize A+T rich sequences collectively called CArG-boxes containing a $CC(A/T)_6GG$ consensus (Shore & Sharrocks 1995).

MADS-box genes have a wide range of functions in the formation of flowers, the control of flowering time, and the control of vegetative development (Riechmann & Meyerowitz 1997, Ng & Yanofsky 2001). The mechanism used to pattern the floral organs is conserved between distantly related flowering plants such as *Arabidopsis* and maize and might have evolved before the divergence of monocots and eudicots. Orthologous genes in different plant species have analogous functions, indicating that the underlying mechanisms that control development might be widely conserved and that MADS-box genes have a crucial function in plant evolution. But certainly, not all MADS-box mutations are homeotic.

Activation of organ identity genes

Floral meristem identity genes, especially *LFY,* play key roles in transcriptional activation of floral organ identity genes (Weigel & Nilsson 1995). LFY protein, a novel transcription factor unique to plants, is first detected in the stage 1 floral primordia and

sustained to stage 7 flowers (Fig 1-7) (Parcy *et al.* 1998, Sessions *et al.* 2000, Wu *et al.* 2000). *LFY* activates *AP1* transcription in all cells of stage 1-2 flower primordia (Wagner 1999). *AP1* will then collaborate with *LFY* to activate class B genes *AP3* and *PI*, as well as class C gene *AG* in stage 3 flowers (Weigel & Meyerowitz 1993, Weigel & Nilsson 1995, Busch *et al.* 1999). Regionalized activation of *AG* in the center of wild-type flowers relies on the integration of flower-specific activities of LFY and meristem-specific activities of WUS (Busch *et al.* 1999, Lohmann *et al.* 2001). *LFY* is sufficient to activate *AG* in strong *wus* mutants while *WUS* is also sufficient to activate *AG* in *lfy* null mutant (Laux *et al.* 1996, Lohmann *et al.* 2001). In wild-type plants, *LFY* and WUS collaboratively activate *AG* via direct binding to the *AG cis*-regulatory elements in a synergistic manner (Lohmann *et al.* 2001).

CAL, an *AP1* homolog, is involved in the activation of another A class gene *AP2* (Kempin *et al.* 1995). Pineiro and Coupland (1998) suggested that *AP1/CAL*, *LFY*, and *AP2* form a positive regulatory loop to maintain their own transcriptions during early flower organogenesis. Floral meristem identity gene *UFO*, encoding an F box protein, facilitates *LFY* in the activation of *AP3* and *PI*. Autoregulation of *AP3* and *PI* maintains their own expression at late flower stages (stage 3 and later) (Goto & Meyerowitz 1994, Honma & Goto 2000, Ng & Yanofsky 2001, Samach *et al.* 1999).

It is understood that the onset of *SEP1*, *2*, and *3* expressions occurs after flower primordia formation but before the onset of B and C class gene expression (Mandel &

Yanofsky 1998, Flanagan & Ma 1994, Savidge *et al.* 1995). However, the regulation of *SEP* genes is unclear.

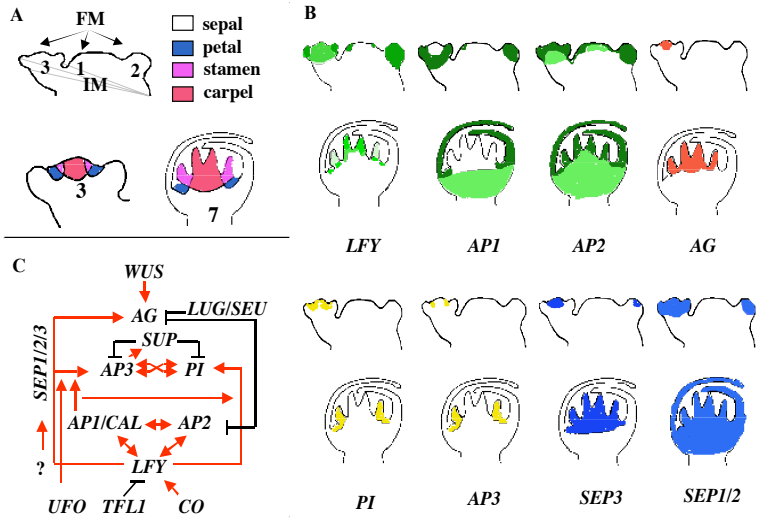


Fig 1-7. Expression patterns and genetic regulatory pathways of floral meristem identity genes and floral organ identity genes. (A) Inflorescence meristem and floral organs. Numbers indicate flower stages. (B) Expression patterns of inflorescence meristem identity genes and floral organ identity genes. (C) Regulatory pathways of inflorescence meristem identity genes and floral organ identity genes.

Cadastral genes

LFY is observed as soon as a flower primordim is formed (Parcy *et al.* 1998, Laux *et al.* 1996, Sessions *et al.* 2000, Wu *et al.* 2000). *AG* transcription, however, is not activated until early stage 3 flowers (Fig 1-7) (Drews *et al.* 1991, Yanofsky *et al.* 1990). These data directly indicate the existence of *AG* negative regulatory factors in stage 1-2 flowers and in the outer two whorls of flowers after stage 3. Indeed, if *AG* is over-expressed, organs in whorls 1 and 2 of flowers will be homeotically transformed to carpelloid and staminoid organs, respectively (Mizukami & Ma, 1992). In fact, all

floral organ identity genes exhibit tissue-specific activities in floral organs. Cadastral genes establish the boundaries of homeotic gene functional activities, despite the fact that some of their expression patterns may not be regionalized (Kunst *et al.* 1998, Dews et al 1991).

SUPERMAN (*SUP*) is a typical cadastral gene that represses B class gene expression in whorl 4 (Bowman *et al.* 1992, Sakai *et al.* 1995, Ito *et al.* 2003). *SUP* encodes a transcription factor and is active in limited regions in stamen primordia and in developing ovaries during flower development. Loss-of-function mutations in *SUP* result in homeotic transformation of carpels to stamens due to the ectopic activities of B class genes in whorl 4.

Floral organ identity genes *AP2* and *AG* act in an antagonistic manner to regulate each other (Bowman *et al.*1991, Drews *et al.* 1991, Sieburth & Meyerowitz 1997). In *ap2* mutants, organs in the outer two whorls are homeotically transformed to carpels and stamens due to ectopically expressed *AG* in whorl 1-2, whereas in *ag* mutants, the inner two whorl of organs are hoemotically transformed into sepals and petals due to the ectopic activities of A class genes in the inner two whorls. Although it is not mechanistically clear, *AP2* represses *AG* possibly in a *LFY*-dependent manner because mutating *AP2* in *lfy* plants did not obviously increase *AG* enhancer activities (Bomblies *et al.* 1999). Interestingly, *AP2* mRNA is distributed in all four whorls of wild-type flowers (Jofuku *et al.* 1994). Therefore, *AG* is unlikely repressing *AP2* at the transcriptional level. Furthermore, recent studies of plant micro-RNAs revealed a new

type of cadastral gene that may shed light on the mechanism of *AP2*-dependent *AG* repression (Aukerman & Sakai 2003, Chen 2003). miRNA172 is highly complementary to *AP2* mRNA in the coding region and is expressed in stage 1 floral primordia and then restricted to the inner two whorls of flowers later. After stage 7, miRNA172 persists in all four whorls. miRNA172 inhibits *AP2* activity at the translational level. Elevated miRNA172 results in losing *AP2* gene function in all four whorls. Therefore, regionalized activities of miRNA172 may explain regionalized activities of *AP2*. Expression of miRNA172 is reduced in *hen1,* a weak genetic enhancer of *ag* (reviewed by Jack 2002). Therefore, *AG* and *AP2* may regulate each other through the mediation of miRNA172.

Two other important *AG* repressors, *LEUNIG* (*LUG*) and *SEUSS* (*SEU*), have been identified (Franks *et al.* 2002, Liu & Meyerowitz 1995). Both precocious (in stage 2 flowers) and ectopic (outer two whorls) expression of *AG* are detectable in *lug* mutants. LUG is a glutamine-rich (Q-rich) protein with seven WD repeats at the C-terminus and a LUFS domain at the N-terminus (Conner & Liu 2000). The LUG protein is localized in the nucleus and has structure motifs (Q-rich domain and WD repeats) present in transcriptional corepressors such as Groucho (Gro) in *Drosophila,* transducin-like enhancer of split (TLE) in human, and TUP1 in yeast (collectively GroTLE family proteins) (Chen & Courey, 2000). GroTLE family proteins repress a number of genes via their association with DNA-binding factors (Dubnicoff *et al.* 1997, Keleher *et al.* 1992, Paroush *et al.* 1994). Once recruited by the DNA-binding factors, GroTLE proteins interact with chromatin modifying factors such as histone deacetylase,

which lead to epigenetic silencing of target genes (Calvo *et al.* 2001, Chen *et al.* 1999, Winnier *et al.* 1999).

Similar to *lug*, mutations in *SEU* cause precocious and ectopic expression of *AG* mRNA (Franks *et al.* 2002), leading to partial homeosis in sepals and petals. Double mutants of *lug seu* exhibit more striking homeotic transformations in outer two whorls and a great extent of flower organ loss. *SEU* encodes a novel protein with two glutamine-rich domains and a highly conserved domain that shares sequence identity with the dimerization domain of the LIM-domain-binding transcription co-regulators in animals. LUG and SEU interact in yeast and *in vitro*, and may form a repression complex that slightly down regulates reporter gene activities in tobacco and *Arabidopsis* protoplast suspensions (Surendrarao, Sridhar and Liu, unpublished data).

However, neither *LUG* nor *SEU* encodes a DNA binding domain. They may repress *AG* expression by mechanisms that are similar to those of GroTEL proteins. That is, the putative LUG/SEU complex may be recruited by DNA-binding transcription factors, which specifically bind to the *cis*-regulatory elements of the target genes, such as *AG*. Therefore, identifying the corresponding a DNA-binding factor(s) may be the key to elucidate *AG* repression mechanisms. This dissertation describes studies in an attempt to identify and characterize such DNA-binding factor. The molecular mechanism that may underlie the *AG* repression in early flower development and organogenesis is discussed in chapters three and four.

TRANSCRIPTIONAL REGULATION

To further justify the studies described in this dissertation, it is important to review the general molecular mechanisms underlying transcriptional regulation in eukaryotic cells. As reviewed by Ottoline Leyser and Stephen Day in their recent book *Mechanisms in Plant Development* (2003), transcription regulations during plant and animal development share maby similarities. The complex multicellularity has evolved twice (three times if you include fungi). Plants provide excellent comparisons with animals for the type of proteins and mechanisms of transcriptional regulatory networks. Plants not only possess transcriptional regulators similar to their counterparts in animals, but also contain unique classes of receptors, transcription factors, and other regulatory components.

Plants provide two unique features for studying the essential roles of transcriptional regulation in development and species survivorship of multicellular organisms. First, plant development is elegantly adjusted to the environmental conditions by transcriptional gene regulations; and second, null alleles of plant developmental regulators are fully viable because the genes are normally acting in postembryonic development, unlike their counterparts in animals, where loss-of-functions are often lethal. This section reviews several aspects of transcriptional regulation that provide important background for "the activation barrier model" proposed in this dissertation.

Arabidopsis genomic perspective

Higher plants have relatively long life spans and low rate of reproduction when compared with fungi. However, unlike animals, plants lack nerve systems and are immobile. Therefore, plants possess minimal ability of mobile avoidance but are exposed to both predictable and unpredictable changes in their life times. Plants thus must possess molecular and genetic means for individual survival upon environmental challenges in addition to basic growth and developmental needs. Three thousand transcription factors or co-regulators (11.8% of the genome, not including 30% unclassified genes) of the *Arabidopsis* genome may fulfill the requirement for both growth and stress responses (AGI 2000). This accounts for two and over three times more transcription factors than those found in *D. melanogaster* and *C. elegans*, respectively, which have similar genome sizes to *Arabidopsis*. Only 8-23% of these factors have counterparts in other eukaryotes. Out of 29 classes of transcription factors, 16 classes appear to be unique to plants. These factors apparently reflect both dependent and independent evolution of transcriptional regulatory mechanisms that may have occurred in plants and other eukaryotes. Nevertheless, transcriptional regulation confers on plants the ability to monitor variations in environmental and developmental cues, to allocate resources and energy efficiently when responding to environmental stresses, to determine the developmental fate of cells, and to maintain basic cell metabolisms and reproduction. Studies of transcriptional regulation using plant systems are challenging yet rewarding to our understanding of basic biology and evolution.

Each family of transcription factor contains multiple members. Some families, such as AP2/EREBP and MADS-box families, consist of up to hundreds of members in *Arabidopsis* (AGI 2000). This inevitably causes functional redundancy among the members of the same family and poses a significant potential barrier to forward genetics or characterizations of gene functions. However, redundancy may be advantageous for plants to integrate target gene functions in response to a vast range of developmental and environmental cues. Furthermore, it may insure plant's survival under genotoxic stresses. Disruption of one copy of the redundant transcription factors may not disrupt the activities of the biological processes with which the gene is involved.

Plants may use transcription factors in different ways in comparison to animals or other eukaryotes. For instance, the proper identity and pattern of body plan alone and the anterior-posterior axis is predominantly controlled by Hox gene family in vertebrates (Patterson & Potter 2003) and arthropods (Hughes & Kaufman 2002). In contrast, MADS-box genes are predominantly involved in the determination of lateral organ identities and the arrangement along the plant and floral centripetal axis (Riechmann & Meyerowitz 1997, Riechmann & Ratcliffe 2003).

Epigenesis

Arising from a single zygote, cells of a plant body are genetically homogeneous. Yet the structures composed of multiple cells and the functions of cell lineages are heterogeneous owing to the transcriptional and posttranscriptional regulation during cell maturation. The transcriptional regulation is achieved through the elegant controls of

repressive and active states of genes, the functional fragments of chromatin. Stable alterations of the transcriptional states are called epigenesis, for they are heritable in the short term, during development and cell proliferation, without alteration in the DNA sequences. Epigenetic states may be maintained through many mitotic divisions or even several meiotic divisions in animals, yeast, and plants (Cavalli & Paro 1998, Cubas *et al.* 1999, Gendall *et al.* 2001, Grewel & Klar 1996, Jacobson & Meyerowitz 1997, Thon & Friis 1997).

Since its introduction in 1940 (Waddington 1940), the concept of epigenesis, "interpretation of the genotype during development to give the phenotype", has evolved. A commonly accepted definition nowadays is the "modifications in gene expression that are brought about by heritable, but potentially reversible, changes in chromatin structure and/or DNA methylation" (Henikoff & Matzke 1997). Such phenomena are found not only in development, but also in genomic defenses to endogenous "pathogenic" DNA and RNA, such as retroelements and transposable elements, whose activities need to be repressed to prevent their duplications in the genome (Hendrich & Bird 1998, Riggs 1975, Strahl & Allis 2000, Volpe *et al.* 2002, Wu & Grunstein 2000). In this thesis, I will exclude genomic DNA methylation that is caused by the presence of foreign DNA or RNA as a type of epigenesis unless the methylation persists after the foreign elements are removed (Matzke *et al.* 2001, Mette *et al.* 2001, Wolffe & Matzke 1999). The reason is that introduction of foreign DNA or RNA is an alteration of genomic contents, even though they may not have been integrated into the chromosomal sequences.

At the molecular level, epigenesis is heritable chromatin modifications, including post-replication DNA methylation/demethylation, addition/removal of covalent histone tail modifications, and interpretation of these modifications (Fig 1-8).

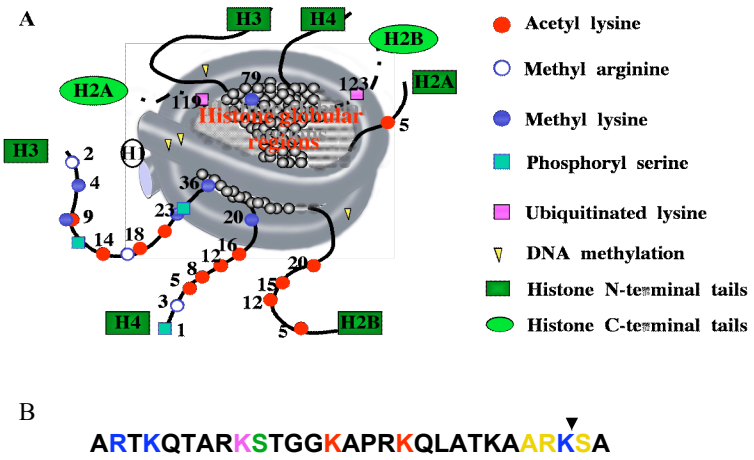


Fig 1-8. Histone modifications and DNA modifications on nucleosome core particle. (A) The N-terminals and C-terminals of histone (histone tails) modifications. Modifications are indicated by colored symbols. The curved tube represents DNA double helix. (B) The N-terminal tail of histone 3. Yellow colored amino residues are located in the nucleosome core particle, but the trypsin-cutting site (arrowed) is accessible on the nucleosome surface. Blue residues can be methylated; red residues can be acetylated; green residues can be phosphorylated; violet residues can be either acetylated or methylated. Modified from Turner (2002).

The first order of DNA folding is the formation of nucleosomes. Each core nucleosome is formed by every ~146 bp of DNA wrapping around a octomer, consisting of one $(H3/H4)_2$ tetramer and two H2A/H2B dimers. A H1 protein binds to the point where DNA enters and exits the core particle to ensure that the DNA wraps two terms around

the core nucleosome. An extra 54-bp of DNA separates every two nucleosomes. Highly conserved N-terminal tails of histones are often covalently modified and are protruding from nucleosomes to provide interactive surfaces for DNA and other *trans*-factors. Types of the modifications are dependent of intrinsic and extrinsic signals and highly correlate with DNA methylation states (Jaenisch & Bird, 2003). The patterns of modifications on histone tails form special sequences (or codes) that can be recognized by *trans*-factors to direct sequential events during transcriptional gene silencing and activation (Schreiber & Bernstein 2002, Agalioti et al 2002)

Chromatin modifications

The *Arabidopsis* genome contains twenty-five methyltransferases distributed on all five chromosomes (AGI 2000). Their regulations, targets, as well as their spatial and temporal specificities in the plant are still unclear. However, the importance of DNA methylation is demonstrated by promoting flowering using chemical demethylation reagent 5-azacytidine (Burn *et al.* 1993). Expressing anti-sense RNA of *MET1* (*METHYLTRANSFERASE1)*, a plant homolog of mouse DNA methyltransferase1, causes genome-wide alteration of DNA methylation patterns that can not be fully restored in the progenies whose transgene has been removed by crosses (Finnegan *et al.* 1998). The transgenic plants exhibit a range of developmental abnormalities including *sup* phenotypes caused by silenced *SUP* and ectopically expressed *AP3* (Finnegan *et al.* 1998, Kishimoto *et al*. 2001). Mutations in SWI2/SNF2 family member *DECREASED DNA METHYLATION 1* (*DDM1*) also cause global hypomethylation and accumulation of phenotypic abnormalities over generations (Vongs *et al.* 1993, Kakutani *et al.* 1996).

Interestingly, in addition to repetitive DNA, single-copy genes are also selectively affected by the *ddm1*, indicating recruitment of this chromatin remodeling factor in a gene-specific manner. Recently, *ARGONUTE 4 (AGO4)*, a gene involved in RNA interference (RNAi) or posttranscriptional gene silence (PTGS), is found to exhibit specificity to certain genes, such as SUP, by methylating both CpNpG and asymmetric sequences except for CpG islands (Zilberman *et al.* 2003). Mutant *ago4* reactivates previously silenced genes such as *sup* alleles via de-methylation. The global effect of *AGO4* is not yet known.

Histone tail modifications are more complicated than DNA methylation/demethylation. Chromatin modifying complexes recruited by transcription factors covalently modify the N-terminal tails of histones by adding or removing phosphate, methyl, mono-ubiquitin, or acetyl groups (Fig 1-8) (Hansen *et al.* 1998, Strahl & Allis 2000, Wu & Grunstein 2000). Sequence similarity and functional profile comparisons among *Arabidopsis*, yeasts, and animals indicated both conservation and diversification on the structures, functions, and usages of proteins involved in these modifications (Jasencakova *et al.* 2003, Manzanero *et al.* 2000, Pandey *et al.* 2002, Springer *et al.* 2003, Wu *et al.* 2000). The *Arabidopsis* genome encodes 16 histone deacetylases (HDAC) and 12 histone acetyltransferases (HAT) (AGI 2000). Computational and phylogenetic analyses suggested that *Arabidopsis* HDACs and HATs have functionally diversified from animals and fungi during plant evolution (Pandey *et al.* 2002). However, similar to other organisms, histone tail modifications closely correlate with DNA methylation/demethylation, and at least in some cases, are

regulated by microRNAs (Volpe *et al.* 2002, Zilberman *et al.* 2003). In both of these studies, H3K9 methylation is an important modification for transcription regulation. It is interesting to notice that the same amino acid residue, H3K9, can be either methylated or acetylated.

Plant histone phosphorylation (S_{10}) during mitosis begins in the pericentromeric chromatin at late prophase and ends at telophase (Houben *et al.* 1999; Kaszas & Cande 2000). In addition, it may have an important role in sister chromatin cohesion during meiosis (Kaszas & Cande, 2000; Manzanero *et al.* 2000). However, a role of histone phosphorylation in transcriptional regulation of plant genes has not been demonstrated.

Addition and removal of methyl, acetyl, and phosphyl groups usually result in transcription states that can be inherited through multiple mitotic divisions. Another type of covalent modification, addition and removal of ubiquitin at the H2B (K_{123}) C-terminal tails, are important for gene activation in yeast (Henry *et al.* 2003). Interestingly, this modification signals to H3 tails in *trans* and alters the modification patterns on H3 tails. Furthermore, ubiquitin modifications seem to only function when the environmental signal is persistently present and does not directly cause cell memories (Henry *et al.* 2003, Shelley L. Berger, The Wistar Institute, University of Pennsylvania, personal communication). Whether ubiquitilation is involved in transcriptional regulation of plant genes is unknown.

Interpretation of chromatin modifications

Interpretation of DNA methylation is unexplored in plant. In mammals, a general rule is that hypermethylation of DNA expels transcription factors from their DNA binding sites and thus represses transcription (Watt & Molloy 1988). However, co-repressors are required to mediate repression in some cases (Billard *et al.* 2002, El-Osta *et al.* 2002, Rietveld *et al.* 2002). In these cases, methyl-CpG binding proteins directly interact with the methylated target DNA and form repression complexes containing HDACs (Feng & Zhang 2001, Nan *et al.* 1998, Ng *et al.* 1999). A lack of methyl-CpG or a lack of methyl-DNA-binding proteins results in de-repression of the target genes. Studies of molecular mechanisms underlying the interpretation of DNA methylation in plants may add another twist to the story.

Studies in animal systems have led to the histone code hypothesis: that distinct patterns of histone tail modifications are recognizable codes for the recruitment of chromatin remodeling complexes, rather than just structural components of chromatin (Strahl & Allis 2000, Turner 2000, Jenuwein & Allis 2001). The best study on how these histone codes are interpreted into repression or activation is the study on human IFN-β gene activation under viral infection (Agalioti *et al.* 2002). This study used reconstituted recombinant nucleosomes bearing amino acid substitution at lysine residues of histone tails. The effects of these mutations on IFN-β gene expression were examined. The results showed that the acetylation of H4K8 is required for recruitment of the SWI/SNF complex, whereas acetylation of H3K9 and H3K14 is critical for the

recruitment of the general transcription factor TFIID. Thus, histone modifications assemble a biochemical cascade that can be interpreted in a sequential manner during transcription initiation. Generally speaking, histone tail methylation, deacetylation, and dephosphorylation lead to repression of genes, whereas histone tail demethylation, acetylation, and phosphorylation usually lead to activation of genes. Both addition and removal of ubiquitin are important for transcriptional activation at different phases of the same stimulus applied to the cell.

In *Arabidopsis*, over-expression of an antisense mRNA of a histone deacetylase antisense RNA blocked AtHDAC expression and resulted in hyperacetylation of histones and consequently pleiotropic effects on development including abnormal shoots and flowers (Tian & Chen 2001). A loss-of-function mutation in the *Arabidopsis* HATs gene (*AtGCN5*) resulted in pleiotropic effects including elevated and ectopic expression of *WUS,* which in turn up-regulates *AG* expression (Bertrand *et al.* 2003). Probably due to the feedback regulations between *WUS* and *AG*, abnormal flower and shoot phenotypes are observed in *Atgcn5* mutants.

Heritability of cell memory

Epigenetic control of gene expression is not limited to animals and fungi. An increasing amount of data shows that plant genes are commonly regulated by the epigenome (Jaenisch & Bird 2003). Over one hundred chromosomal modifying factors, more than in *Drosophila* or yeast, are annotated in the *Arabidopsis* genome (AGI 2000). For example, interacting with DNA binding proteins and chromatin modifying factors,

Polycomb group (PcG) and trithorax group (trxG) complexes maintain the repressive and active states, respectively, in animals (reviewed by Orlando, 2003). Similarly, in *Arabidopsis*, *FLC* (*FLOWER LOCUS C*) expression is down-regulated in response to vernalization and the repressed state is maintained when plants are returned to normal temperature. However, the vernalization initiated repression of *FLC* is no longer maintained when *vernalization2* (*vrn2*) plants are return to normal temperature (Gendall *et al.* 2001). This data suggests that PcG protein VRN2 is involved in the maintenance of epigenetic states over mitotic generations. *VRN2* is not involved in the establishment of epigenetic repressive states, as it is in other biological systems (Gendall *et al.* 2001, Saurin *et al.* 2001). In fact, the repression state of *FLC* is established by VIN3, a homeobox gene encoding a PHD domain protein (Sung & Amasino 2004). *CURLY LEAF (CLF)* is another example of plant PcG genes that are involved in maintaining *AG* repression in vegetative plants. *clf* mutants result in ectopic expression of *AG* in leaves and stems. A plant version of trxG is the *ARABIDOPSIS TRITHORAX-1 (ATX1)* that promotes floral organ formation (Alvarez-Venegas *et al.* 2003). Expression levels of A, B, and C class genes are reduced in *atx1* null mutants.

A key step in maintaining epigenetic state is to inherit the chromatin modification patterns through mitotic cell divisions. In other words, the key is to duplicate the parental DNA modifications to the newly synthesized DNA and to duplicate the histone tail modification patterns of the parental nucleosomes to the newly assembled nucleosomes at the replication fork. Based on existing data, Wolffe & Matzke proposed a mitotic model for future tests (Fig 1-9) (Tyler 2002, Wolffe &

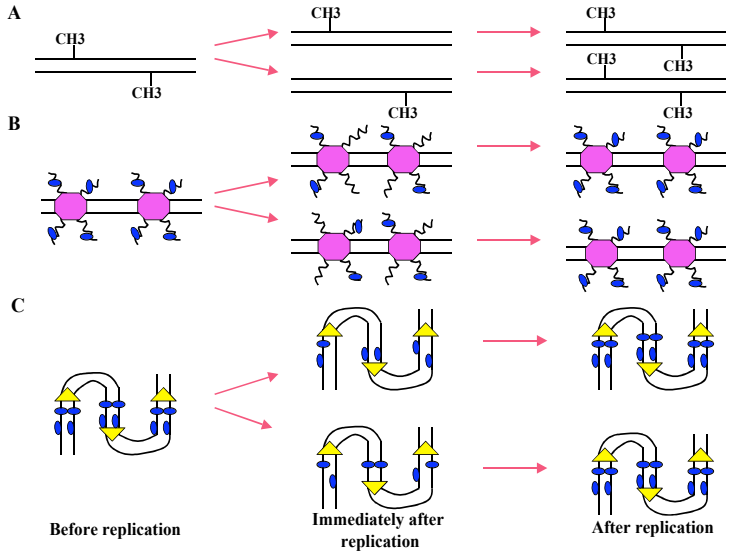


Fig 1-9. Mechanisms mediating the mitotic inheritance of epigenetic effects. (A) Methyl groups modifying symmetrical CpG dinucleotides are randomly distributed to daughter chromatids after replication. This hemimethylated DNA is preferentially recognized by DNA methytransferase sequestered at the replication forks to duplicate parental DNA methylation patterns to daughter chromatids. (B) Newly synthesized histones tails (waving lines) and parentally modified histones (waving lines with blue ovals) are randomly assembled to two daughter chromatids. Histone modifying factors may be randomly segregated to daughter chromatids via their association with the modified histones and are used to modify local nucleosomes. (C) High local concentrations of chromatin modifying factors (blue ovals) localized at DNA repeats (yellow arrows) may facilitate the sequestration of free *trans*-factors from the nucleoplasm onto both daughter chromatids. Modified from Wolffe & Matzke (1999).

Matzke 1999). Three components are proposed to act immediately after DNA replication to duplicate the parental chromatin modifications. First, the symmetric DNA methylation is randomly divided into two daughter chromatids. This

hemimethylated DNA is recognized preferentially by DNA methyltransferase as the substrate for further methylation. Second, free un-modified histones in nucleoplasm and parental modified histones will be randomly assembled to the daughter chromatids by histone chaperones in an ATP-dependent manner. Histone modifying factors that are associated with the modified histones will modify the local chromatin. Third, highly concentrated *trans*-acting factors at repetitive DNA may facilitate the sequestration of free chromatin modifying factors from nucleoplasm onto chromatids after replication. Overall, post-replication chromatin modification machineries may act under the guidance of pre-existing modifications that are semiconservatively (in a loss sense) obtained from parental chromatins.

Transcription initiation

Activation of human IFN-β gene is a well-documented example of transcription initiation. Gene activation starts from cooperative binding of gene specific transcription activators and the activator-recruited coactivators to the hypomethylated nucleosome-free zone of enhancer DNA to form an enhancersome (Merika & Thanos 2001). This enhancersome guides the modification and repositioning of the nucleosome that has been blocking the formation of a transcriptional pre-initiation complex on the promoter. To achieve this, the enhancersome recruits HATs-containing complex that will acetylates the nucleosome to be repositioned (Agalioti et al 2001). CBP-Pol II holoenzyme complex is then recruited to the enhancersome to stabilize the subsequent recruitment of SWI/SNF complex, via the guidance of histone codes (Munshi et al 2001). The SWI/SNF complex further alters the nucleosome structure via ATP-

dependent mechanisms and induces DNA binding by TFIID (Merika & Thanos 2001), which will in turn induce DNA bending and sliding of the nucleosome toward downstream for about 36 nucleotides (Lomvardas & Thanos 2001, Peterson 2002). Thus far, the transcription preinitiation complex is formed and transcription is about to occur.

CONCLUSION

Transcriptional regulations play important roles in cell fate determinations and body part patternings in plants. Plant genomes, such as in *Arabidopsis thaliana*, contain features that are commen to all eukaryotes and features that are unique to plants. Studies the transcription factors and transcriptional mechanisms in plants do not only increase our freedom in utilize the natural resourses from plants, but also shed lights on the general mechanisms underlying the development and evolution of multicellular organisms.

Transcriptional regulation of *AG* is one of the most well documented examples in *Arabidopsis*. The attention of developmental biologists toward *AG* may have well reflected the importance of this gene. First, *AG* is one of the prototype of MADS box genes. Many *AG*-like genes have been found in plant genomes and found are involved in a variety of biological processed. Study *AG* may provide useful information that helps understanding of the functions and regulations of other MADS box genes. Second, the transcription activities of *AG* coincide with the translation and thus the functional activities of the gene. Third, *AG* regulates formation of the organs that

support the gemetaphytes generation of the plant. Therefore the regulations and functions of *AG* are directly exposed to natural selection. And last, not the least, florists and agronomists are very much interested in improving the diversity of flower morphology and the yields of seeds and fruits, both of which are related to the functions of *AG* and it homologs. However, despite the all the efforts accumulated in the past 15 years, the repressive mechanism that defines the boundaries of *AG* activities is still elusive. This thesis describes studies on a novel *AG* repressor, LARSON (LSN), which directly binds *AG* enhancer elements *in vitro*. Interestingly, the spatial expression of *LSN* did not explain the spatial repression patterns of *AG*. Data suggested that the temporal activities of *LSN* might play important roles in establishing *AG* repression in the ancestoral cells of organ primodia in the inflorescence and floral meristem peripherial zones. This repression was probably maintained by machineries act in cells of organ primordia in both inflorescence and floral meristems.

LSN is not only the first *AG* repressor that has been found to date to bind *AG cis*-elements, but more importantly, it suggests a novel rationale for understanding body part patterning and cell fate determination. That is, the spatial specific regulation, as described by the ABCE model, is not the only way to determine spatial patternings of body structures. The temporally acting factors can define the developmental history of cells and, thus, determine the developmental fates of cells and the organ types that the cells are to build up.

Chapter two

ISOLATION AND CHARACTERIZATION OF *LARSON (LSN)*

ABSTRACT

To further understand how the negative regulatory fractors interact with *AG cis*-elements and how the tissue specificity of the negative regulatory machinery is determined, I isolated and characterized an novel *AG* repressor, *LARSON* (*LSN*). LSN is a homeodomain protein that directly binded to *AG cis*-elements *in vitro*. *In situ* hybridization data suggested that *LSN* was transiently expressed in cells located in theperipheral zone of inflorescence and floral meristems immidiately before they were specified to organ primordia. Partial homeotic transformation of shoot apical meristem in *lsn* mutant plants suggested that *LSN* was a key factor in establishing *AG* repression in the multipotential cells before they were committed to specific organ primordia. Significant reduction of *LSN* expression in the sepal primodia in stage 3 flowers suggested that the maintenaince of *AG* repression might not require LSN.

INTRODUCTION

Patterning of plant body parts

It has been proposed that "the variety of plant forms are simply different modifications of a common growth plan" (Coen & Carpenter 1993). Comparisons of a flower and a shoot are considered (by assessing organ identity, internode length, phyllotaxy, and determinacy) the best illustration of such a unifying theory. Floral meristem is said to be the equivalent of inflorescence meristem and floral organs are the

equivalent of leaves. *Arabidopsis* inflorescence meristem and floral meristem share essentially the same cytohistological zonation patterns and genetic control of cell divisions and cell organization in the meristem (Clark 1997, Coen & Carpenter 1993, Meyerowitz 1997). Both floral and inflorescence meristems are organized in tunica-corpus duplex structures superimposed with three distinctive L1, L2, and L3 cell layers. Cells divide anticlinally in L1 epidermal cell layer and divide in all planes the inner L2 and L3 layers in the central zone to provide pluripotential cells to the peripheral zone that surrounds the central zone in a doughnut shape (Fig 1-2, Fig 3-1A). The peripheral

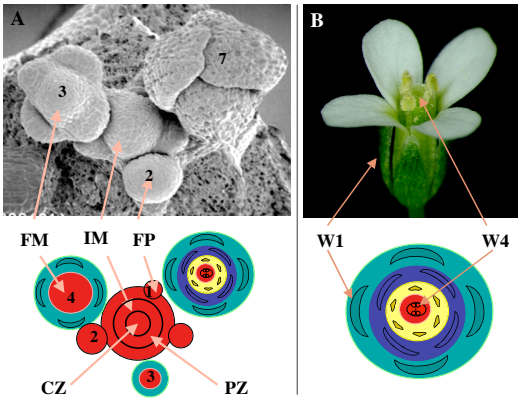


Fig 2-1. Meristem organization and functions. (A) An inflorescence meristem (IM) produces floral primordia (FP) in a spiral pattern. (B) A floral meristem (FM) produces floral organ primordia that are arranged in four concentric whorls (W). Numbers indicate the stages of flower primordia. CZ, central zone; PZ, peripheral zone.

zone contains organ initial cells (or organ progenitors) that will further divide and differentiate into specific cell types composing organ primordia (Meyerowitz 1997). Further divisions and specification of primordial cells form different types of lateral organs. At the mean time, expansion and cell divisions in the rib zone underneath the central zone pushes the meristem upward, leaving the lateral organs behind. The

functional differences between floral and inflorescence meristems are determined by the differentially acting meristem identity genes. Therefore, ectopic expression of floral meristem identity genes in the inflorescence meristem may predict homeotic transformation of inflorescence to floral meristem.

Genetic control of positioning and timing of the initial cells specifies organization and patterning of plant body parts, whereas tissue-specific gene expression in the organ primordia determine the identity of organ types (Clark 1997, Parcy *et al.* 1998). *Arabidopsis* inflorescence meristem produces flowers that are arranged in a spiral pattern along the axis of stems. Cells in the floral meristem assume distinctive identities and form four types of floral organs arranged in four concentric whorls. From the outermost to the innermost, there are four sepals in whorl 1, four petals in whorl 2, six stamens in whorl 3, and two carpels, which are fused to form a gynoecium, in whorl 4 (Fig 1-6, Fig 2-1B). The identities of these organs are determined by a small group of homeotic genes summarized into the A, B, C, and E classes (Fig 1-6) (Bowman *et al.* 1989, Bowman *et al.* 1991, Coen & Meyerowitz 1991, Honma & Goto 2001, Okamuro *et al.* 1993, Pelaz *et al.* 2000, Meyerowitz *et al.* 1991, Weigel & Meyerowitz 1994). These genes may act alone or cooperatively to turn on organ specific developmental programs.

How cells in a multicellular organism assume their developmental fates and form distinct patterns is a fundamental biological question. To address this question, I studied genetic and molecular regulation of *Arabidopsis* flower organ formation and

identity determination. More specifically, how the regionalized *AGAMOUS (AG)* expression is realized during flower organogenesis.

Functions of *AG*

AG is a MADS-box transcription factor and is directly activated by *LFY* and *WUS* in the inner two whorls of flowers starting at stage 3 when sepal primordia are initiated (Busch et al 1999, Drews *et al.* 1991, Lohmann *et al.* 2001, Yanofsky *et al.* 1990). *AG* represses A class flower organ identity genes in the inner two whorls (Bowman *et al.* 1989, Bowman *et al.* 1991). In *ag* loss-of-function mutants, stamens are converted into petals and carpels into multiple repeats of (sepals-petals-petals)n, a direct evidence of ectopic activities of A class genes in the inner two whorls. The repetitions of (sepals-petals-petals)n, namely indeterminacy, is due to extended expression of *WUS* after stage 6 in *ag* flowers, at which time *WUS* expression is normally terminated by *AG* in wild-type flowers (Lenhard *et al.* 2001). The indeterminacy of *ag* floral meristem is not only evidenced by an increased number of whorls of flower organs, but also by faciation of FM at later floral stages. A faciated FM produces multiple secondary *ag* flowers on the primary petiole, resembling a shoot. These *ag* phenotypes indicate that *AG* is functioning in repressing meristematic cell proliferation at the floral meristem. On the other hand, ectopic and elevated *AG* expression in floral organs is sufficient to transform sepals to carpels and petals to stamens, but insufficient to transform leaves to floral organs (Mizukami & Ma 1992).

Epigenetic regulation of *AG* expression

CURLY LEAF (CLF) and *ARABIDOPSIS TRITHORAX-1(ATX1)* act in an antagonistic manner to regulate *AG* expression in *Arabidopsis* (Alvarez-Venegas *et al.* 2003, Goodrich *et al.* 1997). CLF belongs to ESC (extra sex combs) type PcG (polycomb group) protein family that is known to maintain gene silencing through mitotic cell divisions. How PcG complexes find their way onto chromatin and convey epigenetic repression is still not clear. However, it has been shown that fusing a DNA-binding domain to a PcG protein can trigger target gene repression, which, however, cannot be maintained (Poux et al 2001). Therefore, another repression complex, presumably a repression initiation complex, may be required to prepare the chromatin for PcG complexes. *CLF* is thought to maintain *AG* repression state in vegetative plant tissues (Goodrich *et al.* 1997). Mutating *CLF* leads to *AG* transcription in leaves and flower buds, presumably by relaxing the chromatin structure at the *AG* enhancer region. ATX1 belongs to trxG (trithorax group) protein family that maintains the activation states of genes. ATX1 contains a SET (SU(var)3-9, E(Z), and trithorax) domain that has a histone H3-specific methytransferase activity *in vitro*, suggesting its ability to remodel chromatin structures (Alvarez-Venegas *et al.* 2003). *ATX1* transcripts are observed in inflorescence meristem and are especially elevated in floral primordia in wild-type *Arabidopsis*. *atx1* knock-out plants produce abnormal flowers due to reduced expression levels of floral homeotic genes, including *AG* (Alvarez-Venegas *et al.* 2003). Over-expression of *ATX1,* a histone methylase, elevates *AG* expression levels but does not alter the spatial expression patterns of *AG* in the early stages of flower organogenesis. Thus, *ATX1* is proposed to provide transcriptional competence to the

AG enhancer so that *AG*-specific transcription factors can access their binding sites and recruit co-activators for transcription initiation.

Epigenetic regulation of genes often relies on histone tail modifications. In *Arabidopsis*, over-expressing antisense RNA of *AtHDAC* results in hyperacetylation of histones and consequently pleiotropic effects on development, including abnormal shoots and flowers (Tian & Chen 2001). A mutation in an *Arabidopsis* HATs gene (*AtGCN5*) results in pleiotropic effects including elevated and ectopic expression of *WUS* that, in turn, up-regulates *AG* expression (Bertrand *et al.* 2003). Probably due to feed back regulations between *WUS* and *AG*, abnormal flower and shoot phenotypes are, therefore, observed in *Atgcn5* mutants.

The *AG* enhancer DNA

LFY activates *AG* via direct binding to a *cis*-regulatory element, located at the 3' of the second intron of *AG* (Busch *et al.* 1999, Lohmann *et al.* 2001). WUS binding site (WBS) is 1 bp down stream of the LFY binding site (LBS), synergistically activate *AG* (Fig 2-2) (Lohmann *et al.* 2001). LFY and WUS actions, however, cannot explain the elegant spatial and temporal patterns of *AG* expression. For instance, immunolocalization showed that LFY protein is present in stage 1-2 flower primordia where *AG* is not expressed (Parcy *et al.*, 1998). Introduction of a strong activator *LFY*:*VP16,* driven by the *LFY* enhancer DNA, to wild-type plants can activate *AG* ectopically in the locations where *LFY:VP16* is expressed, including in stage 1-2 flowers. Thus, LFY alone is insufficient to activate *AG* before stage 3 flowers and in

the outer two whorls of flowers in wild-type plants. In addition, replacing *AG* enhancer with the stronger 35s-CaMV promoter, that is not specifically repressed by *AG* repressors, cause ectopic expression of *AG* and thus growth of carpels in whorl 1 and ectopic stamen in whorl 2 (Mizukami & Ma, 1992). Therefore, a repression complex recognizing specific *AG cis*-regulatory element(s) is predicted to repress *AG*, assuming the role of CLF complex in the inflorescence meristem and stage 1-2 flowers. The *AG* repression state may be maintained in the outer two whorl floral organs after stage 2 when sepals and stamens are initiated.

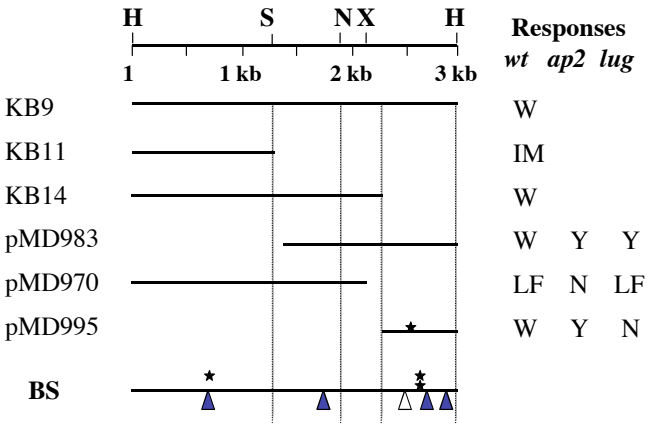


Fig 2-2. Activities of *AG* enhancers. based on *GUS* reporter gene systems. Names of transgenic lines are listed on the left. Activities and responses of *AG* enhancers are listed on the right. The scale indicates sequence length and is labeled with restriction sites (H, Hind III; S, Spe I; N, Nla IV; X, Xba I). *AG cis*-regulatory elements that are conserved in Brassicaceae species are indicated on the bottom. Open triangle is predicted for repression maintenance and filled triangles are important for activation and maintenance. Asterisks indicate predicted or proven LFY and WUS binding sites. BS, binding sites; IM, inflorescence meristem; LF, late flowers (> stage 5); N, no; W, wild-type pattern; Y, yes. Modified from Busch et al 1999, Deyholos & Sieburth 2000, Hong *et al.* 2003.

Transgenic plants containing *GUS* reporter gene systems (various fragments of *AG* enhancer driven β-glucuronidase) have been established to study *AG* regulation (Fig 2-2) (Busch *et al.* 1999, Deyholos & Sieburth 2000, Hong *et al.* 2003, Sieburth and Meyerowitz 1997). The expression patterns of *GUS* gene driven by different *AG* enhancer sequences reflect regulatory functions of each *cis*-regulatory element. Furthermore, the *AG::GUS* reporter expression patterns can be examined in different mutant plants to determine the effects of each mutated gene on *AG* expression. A difference (or a response) between the activities of an *AG* enhancer DNA fragment in wild-type and mutant plants indicates that the wild-type gene corresponding to the mutant acts through this *AG* enhancer.

The second intron (~3 kb) of *AG* contains multiple *cis*-regulatory elements (Busch *et al.* 1999, Deyholos & Sieburth 2000, Hong *et al.* 2003, Sieburth and Meyerowitz 1997). The reporter gene system containing the transcriptional fusion of this intronic DNA with *GUS* is designated KB9 (Fig 2-2). Spatial and temporal activities of KB9 reporter system completely mimic regionalized mRNA distribution of *AG* during early flower organogenesis detected by *in situ* hybridization. Although the 3' and the 5' ends of the intronic DNA show certain redundancy, the 3' enhancer is more important for the early activation of *AG* and expression in carpels, whereas the 5' enhancer appears to be more important for late *AG* expression in stamens (Deyholos & Sieburth, 2000). Hong *et al.* (2003) attempted to identify more *cis*-regulatory elements using phylogenetic footprinting and shadowing. In this study, invariant motifs among Brassicaceae species (phylogenetic shadowing) and non-Brassicaceae species

(phylogenetic footprinting) were identified by aligning the second intronic sequences from the orthologues of *AG*. These invariant motifs were tested for their regulatory functions using transcriptionally fused *GUS* reporter in *Arabidopsis* plants. This study identified 4 highly conserved activation and maintenance motifs, including putative LFY and WUS binding sites, and 1 repression maintenance motif. Notably, positioning of LFY and WUS binding sites are highly variable in Brassicaceae. The previously identified LFY and WUS binding sites located at the 3' of *AG* second intron by EMSA were missed in this assay (Hong et al 2003, Busch *et al.* 1999, Lohmann *et al.* 2001). Furthermore, no *cis*-element has been yet found that act to initiate repression, presumably due to lack of mutants involved in flower specific initiation of *AG* repression.

Negative regulation of *AG*

Since *AG* is activated only in a subset of *LFY*-expressing cells, region-specific regulators must be required either to activate *AG* in the inner two whorls of stage 3 flowers, or to repress *AG* before stage 3 and in the outer two whorls of older flowers, or both. Several *AG* negative regulators have been identified and their regulatory functions will be reviewed in chapter four. It is, however, necessary to mention that the *AG* second intron also responds to the regulation by two negative regulators, *AP2* and *LUG* (Fig 2-2) (Deyholos & Sieburth 2000, Bomblies *et al.* 1999, Liu & Meyerowitz 1995, Drews & Bowman 1991). Wild-type and *ap2* mutant plants carrying pMD970 reporter transgene showed the same GUS staining patterns in flowers, indicating that *AP2* activities are not important for regulating the *AG* enhancer in pMD970. Wild-type

and *ap2* mutant plants carrying pMD995 reporter, in which *GUS* is driven by the 3' sequences of *AG* intron, showed different GUS staining patterns. GUS is constrained in the inner two whorls of wild-type flowers but not in *ap2* flowers. Therefore, *AP2* acts through the *cis*-regulatory elements presented in pMD995 enhancer (Bomblies *et al.* 1999, Deyholos & Sieburth 2000). Using similar analyses, Bomblies *et al.* (1999) suggested that *AP2* is dependent upon the LFY-binding site (LBS) when it represses *AG*. In this assay, the LBS was deleted from an *AG* enhancer sequence that was slightly shorter than pMD995 at the 5' end (Fig 2-2). This reporter gene system was not responding to *LFY* because it showed reduced GUS staining in *lfy* mutant plants or *LFY*:*VP16* plants compare to in wild-type plants. This reporter gene system slightly responded to *ap2* mutant as indicated by slightly increased GUS staining in *ap2* flowers. However, if the LBS was not deleted, the response to *ap2* mutant was strongly attenuated. Whether *AP2* directly binds to *AG* enhancer DNA to regulate *AG* is still not yet known.

The co-repressor *LUG* also acts on the enhancer in the second intron of *AG*. Both pMD983 and pMD970, but not pMD995, respond to *lug* mutants. Thus, *LUG* likely acts through *cis*-regulatory element(s) located in the overlapping region of pMD983 and pMD970 enhancers (Deyholos & Sieburth 2000).

Both *AP2* and *LUG* are expressed in all four whorls of flowers and in inflorescence meristem (Conner & Liu 2000, Jofuku et al 1994). The region specific repression of *AG* cannot be explained by *AP2* and *LUG* expression patterns. Although

continuous research has identified more negative regulators of *AG*, none has been shown to directly bind to *AG* enhancer. Specific repressive *cis*-regulatory elements in the *AG* enhancer have not yet been identified.

This chapter describes the isolation and characterization of a novel *AG* repressor *LARSON* (*LSN*). LSN is a homeodomain protein and directly binds to *AG cis*-elements residing in the second intron of *AG*. The regionalized expression pattern of *LSN* suggests that *LSN* acts in the meristem peripheral zone conveying mitotic imprints on *AG* enhancer activities. Specifically, *LSN* mRNA is detected in both inflorescence and floral meristems and peaked in the pre-primordial cells in the peripheral zone. A *lsn-1* missense mutation leads to ectopic expression of *AG* in inflorescence meristem leading to the formation of ectopic carpels at the shoot apex. Although *LSN* transcripta are not detected in the sepal primordia, lack of *LSN* activities in the floral meristem peripheral zone in *lsn-1* plants lead to ectopic *AG* expression in sepals causing homeotic transformation of sepals to carpels. The possible mechanisms underlying *LSN* functions in *AG* repression are discussed.

MATERIAL AND METHODS

(see chapter five)

RESULTS

lsn-1 showed normal morphology during early reproductive phase

Under normal growth conditions (20 ^{o}C, continuous light, 50% humidity), reproductive *lsn-1* plants grow slower than wild-type L*er* plants (Fig 2-3A). *lsn-1* and wild-type plants eventually grow to approximately the same height when grown older. The inflorescences of *lsn-1* plants are similar to those of the wild-type L*er* plants and produce normal flowers during early reproductive phases (Fig 2-3B-E) (See quantitative details in chapter three). These morphological characteristics do not change if plants are subjected to short-days (light:dark = 8:16). Flowering time of *lsn-1* plants was similar to that of wild type L*er* plants. L*er* and *lsn-1* plants start to flower at 8.6 and 8.5 (n = 24) rosette leaves at continuous light, respectively. They start flowering at 33.1 and 32.4 (n = 20) leaves under short-day condition, respectively (Fig 2-3 F, G).

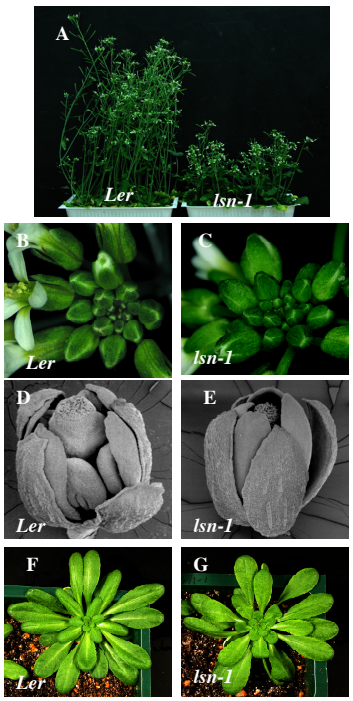


Fig 2-3. *lsn-1* may produce normal flowers in the early reproductive phase at 20 ^{o}C under both long and short day conditions. (A) A comparison between L*er* and *lsn-1* growth. (B) A L*er* inflorescence. (C) A *lsn-1* inflorescence. (D) A L*er* flower. One sepal was slightly damaged during handling. (E) A *lsn-1* flower. (F) A L*er* plant grown at short-day (8 hour light/16 hour dark). (G) A *lsn-1* plant grown at short-day.

lsn-1 has abnormal phyllotaxy

lsn-1 plants showed abnormal phyllotaxy at all conditions tested (Fig 2-4). Wild-type inflorescences generated flowers, at a regular internode length, arranged to a spiral pattern with an average of 137.5^{o} between two consecutive flowers. In *lsn-1*, however, more than one flowers or secondary shoots may be produced at one node. Up to 1-3 primordia may grow at the same node. Multiple floral primordia at the same node often made the *lsn-1* inflorescence appeared larger than that of wild-type L*er* plants.

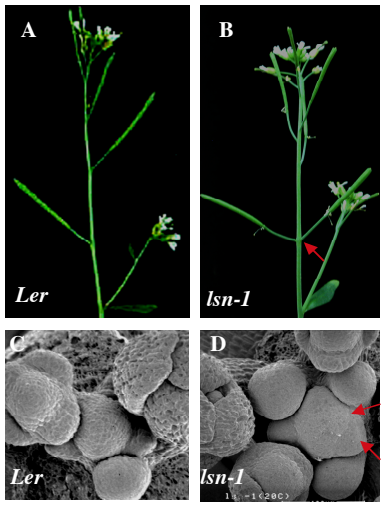


Fig 2-4. *LSN* controls phyllotaxy of *Arabidopsis* plants. (A) A shoot of wild-type L*er* plant. (B) A shoot of *lsn-1* plant. (C) The inflorescence meristem of L*er* plant. (D) The inflorescence of *lsn-1* plant. Red arrows indicate flowers or flower primordia produced at abnormal phyllotactic positions.

High temperature enhanced *lsn-1* flower phenotypes

A small portion of *lsn-1* plants produced abnormal flowers at the late reproductive phase (Fig 2-5A, B). This phenotype was enhanced by growth at high environmental temperature (29 ^{o}C). Specifically, more plants produced abnormal flowers and each plant produced more abnormal flowers (see chapter three for

quantification). These abnormal flowers showed fused or partially fused carpels and are subtended by a carpelloid bract or filament (Fig 2-5B). Under scaning electron microscope (SEM), cross sections of the abnormal flowers showed that the fused or partially fused carpels were homeotically transformed first whorl organs. Stigmatic tissues were found at the tip of these first whorl carpels (Fig 2-5B). Ovules were grown at the margins of carpel valves (Fig 2-5 C). Three to four carpels were often produced in the first whorl (Table 2-1). Septum as well as transmitting tissue development were often incomplete. Organ numbers of the inner three whorls were significantly reduced (Table 2-1). Whorl 2 organs were usually absent. Variable numbers of whorl 3 organs were observed. Pollen of the abnormal flowers seemed abnormal and failed to fertilize normal carpels (data not shown) when they are used to test paternal imprints of the abnormal floral phenotypes. Whorl 4 organs were either absent or grown with incompletely formed septums (Fig 2-5C, D) (Table 2-1).

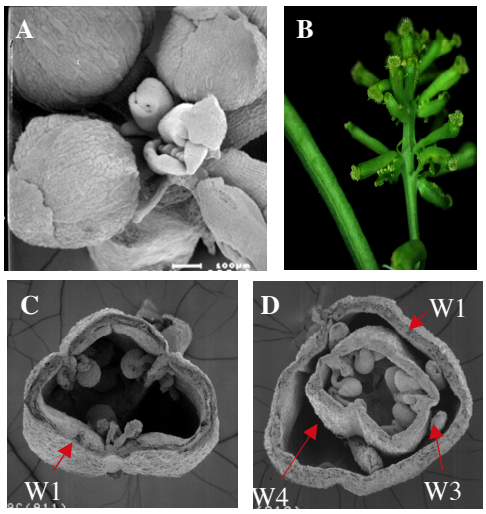


Fig 2-5. Homeotic floral phenotypes of *lsn-1* at 29 ^{o}C. (A) A *lsn-1* inflorescence beginning to produce abnormal flowers. (B) A *lsn-1* shoot bearing multiple abnormal flowers. (C) The cross-section of a *lsn-1* abnormal flower showing three fused carpels in whorl 1 (w1). (D) The cross section of a *lsn-1* abnormal flower showing fused whorl1 and whorl 4 (W4) carpels and a reduced number of whorl 3 stamens.

Table 2-1. Flower organ count in two types of *lsn-1* flowers.

	Organ numbers per flower (n= 20)			
	Whorl 1	**Whorl 2**	**Whorl 3**	**Whorl 4**
Early flowers	4.0 ± 0.00	4.0 ± 0.00	6.0 ± 0.00	2.0 ± 0.00
Abnormal flowers	3.6 ± 0.46	0.0 ± 0.00	1.4 ± 1.38	1.0 ± 1.22

Flowers (stages 11-13) from *lsn-1* plants were collected and dissected under light microscope.

Abnormal flowers were produced from the shoot apex

Most interestingly, close observation of *lsn-1* inflorescence meristem revealed more abnormal phenotypes when plants were grown at 29 ^{o}C (Fig 2-6). In all the samples studied, the inflorescence was partially transformed into a floral meristem that produced congenitally fused carpels, designated apical carpelloid flower (acf), at the shoot apex (Fig 2-6 A, B). A filament may subtend the acf (Fig 2-6C).

The acf did not terminate the growth of shoots or terminate the production of more flowers. More carpelloid flowers were continually grown at the receptacle of the acf, either from outside of the acf (Fig 2-6 C) or inside of acf (Fig 2-6 D), judged by the maturation stages of the carpelloid flowers. As more carpelloid flowers or carpelloid structures were produced at the apex, old flowers were left behind forming an inflorescence bearing multiple abnormal flowers not only at the apex, but also lateral along the stem (Fig 2-5B Fig 2-6E). All the capelloid flowers, including the acf, were collectively named terminal carpelloid flowers (tcf) because they were produced after producing normal flowers in the early reproductive phase. Occasionally, a partially

transformed inflorescence meristem produces under-developed stamens that subtend the acf (Fig 2-6E). Morphologically normal ovules were often found on the carpelloid flowers (Fig 2-6G).

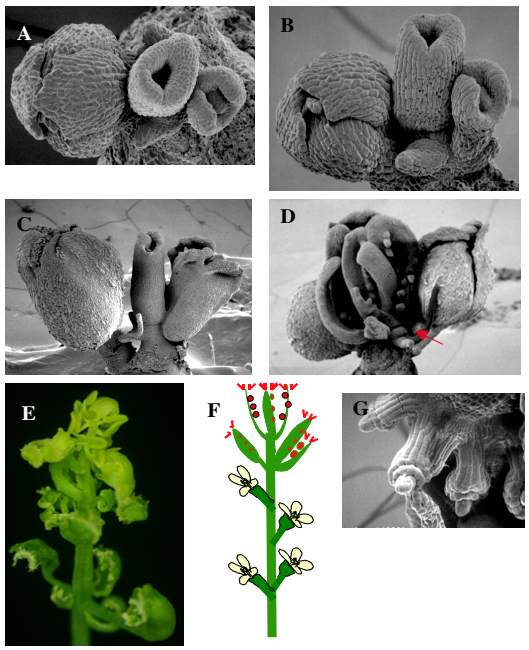


Fig 2-6. Partial homeotic transformation in the *lsn-1* inflorescence meristem. (A) The overhead of an apical carpelloid flower (acf). (B) The side view of the acf in A. Note the normal flower left to the acf and an early stage abnormal flower on the right. (C) The side view of an acf. Note that a filament was subtending the acf on the left and an abnormal flower subtended by carpelloid bracts on the right. (D) The side view of a shoot apical meristem topped with carpelloid structures. A piece of carpelloid structure was dissected indicated by the red arrow. Morphologically normal flowers flanked the acf. (F) Summary of *lsn-1* shoot phenotype. (G) Ovules grown on carpelloid organs.

The transition from producing normal flowers to producing abnormal flowers was very rapid in *lsn-1*. Flowers on the same *lsn-1* shoot were either normal or abnormal. Intermediate flower phenotypes were rarely observed (Fig 2-5 A, Fig 2-6 A-D, Fig 2-7, Fig 2-9). Every shoot that bore tcf, either studied under SEM or light microscopy, had an acf.

AG was ectopically expressed in *lsn-1* plants

The similarities between *lsn-1* flower phenotypes and the phenotypes of other known *AG* negative regulators led to the examination of *AG* expression in *lsn-1* plants. To do this, KB9 transgenic plants were crossed into *lsn-1* plants. The *AG* enhancer of KB9 construct was both necessary and sufficient to drive *GUS* expression in a pattern identical to the wild-type *AG*. When plants were grown at 29 ^{o}C, KB9 was activated with wild-type *AG* expression patterns in *lsn-1* flowers during early reproductive phase. For example, *GUS* activities were restricted to the inner two whorls of flowers starting at stage 3 (Fig2-7A).

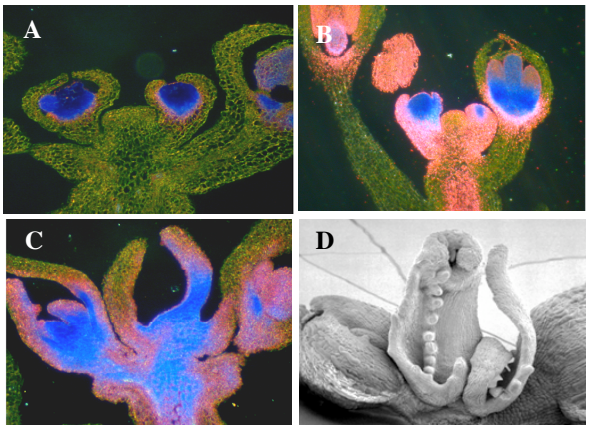


Fig 2-7. KB9 was ectopically activated in *lsn-1* plants at 29 ^{o}C. (A) GUS staining at early reproductive phase. (B) GUS staining at late reproductive phase. (C) Attenuated *GUS* expression at the late reproductive phase when acf were formed. (D) A apical carpelloid flower similar to C.

As the plant grew older, *GUS* was expressed ectopically in petal primordia, sepals, receptacle, and petiole (Fig 2-7B). More strikingly, the KB9 was precociously activated in the shoot apex and in stage 1-2 flower primordia. The flower primordia showed a stronger signal than that in the shoot apex. When the inflorescence meristem produced acf, *GUS* activities became intensified (Fig 2-7C). The relative levels of *GUS*

expression correlated closely with the morphological abnormalities in the *lsn-1* inflorescence meristem and flowers. These observations suggested that the ectopic and precocious *AG* activities might be the cause of the homeotic transformations observed in *lsn-1* flowers and shoots at 29^{o}C.

Homeotic transformation in *lsn-1* was caused by ectopic *AG*

The GUS staining patterns suggested that ectopic *AG* expression in the flowers and inflorescence meristem of *lsn-1* was likely responsible for the homeotic phenotypes observed in *lsn-1* plants. To determine whether ectopic *AG* was mediating *lsn-1* phenotypes in flower organ identities, *ag-1 lsn-1* double mutant plants were constructed and analyzed. Overall, *ag-1 lsn-1* floral morphology resembled that of the *ag-1* flowers at 29 ^{o}C (Fig 2-8A, C). Four sepals were developed in whorl 1 of *ag-1 lsn-1* mutant plants. Whorl 2, 3 and 4 organs were petals, petals, and repeats of "sepal-petal-petal", respectively, as they were in *ag-1* single mutants. The homeotic transformation of first whorl organs as well as reductions of floral organ numbers in whorls 2-4 in *lsn-1* plants were suppressed in *ag-1 lsn-1* plants. In other words, abolishing *AG* functions in *lsn-1* plants removed homeosis in flowers. The acf phenotype was not produced in *ag-1 lsn-1* plants. These phenotypes indicate that the partial conversion of inflorescence meristem to floral meristem in *lsn-1* plants was caused by ectopic *AG* activities. Thus, *ag-1* was epistatic to *lsn-1,* and ectopic *AG* mediates *lsn-1* phenotypes.

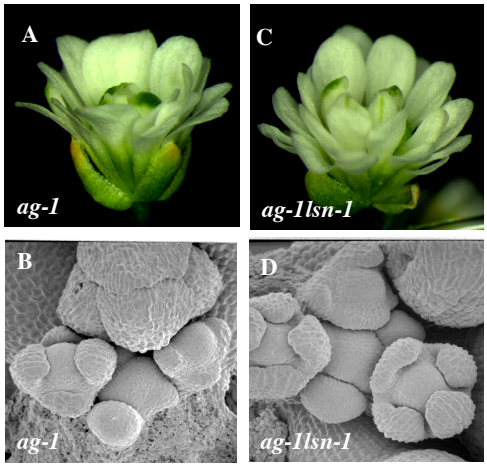


Fig 2-8. *AG* mediates floral and infloresence meristem abnormalities in *lsn-1* plants at 29 ^{o}C. (A) An *ag-1* flower. (B) An *ag-1* inflorescence meristem. (C) An *ag-1 lsn-1* flower. (D) An *ag-1 lsn-1* inflorescence meristem.

LSN determines phyllotaxy in *Arabidopsis* plants

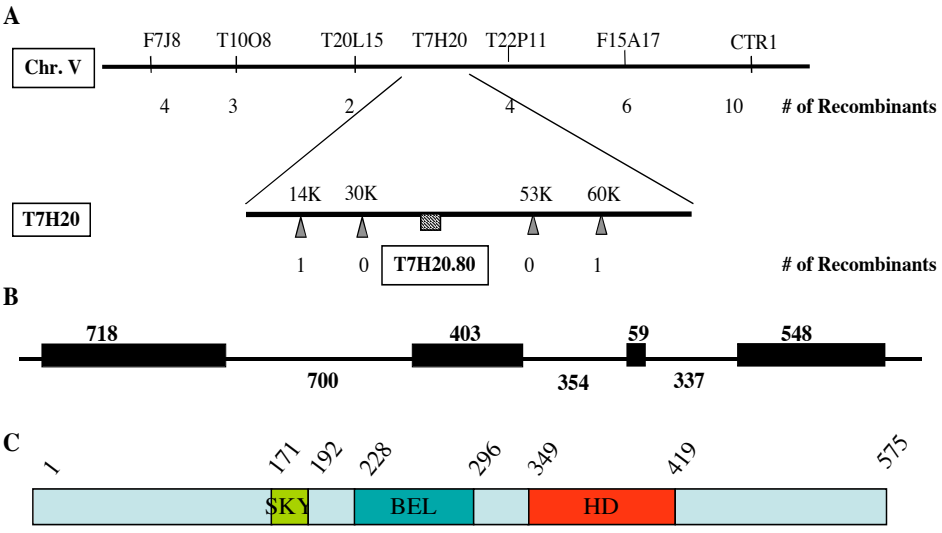
To determine whether all of the *lsn-1* phenotypes are mediated by ectopic *AG* activities, phyllotaxy was compared among different plant genotypes. The phyllotaxy abnormalities tightly co-segregated with the *lsn-1* mutation either in the presence or absence of *ag-1*. Furthermore, the temperatures under which the plants were grown did not affect the severity of the *lsn-1* phyllotaxy defect (Fig 2-9). Specifically, more than one flower might be produced at the same node (2-9 A, C) in *lsn-1* and *ag-1 lsn-1* plants, but not in *ag-1* plants (Fig 2-9B). Thus, ectopic *AG* did not mediate the phyllotaxy abnormality caused by *lsn-1*. *LSN* determines the position and timing of initial cells in the inflorescence meristem independent of *AG*.

Fig 2-9. *LSN* determines the positions of initial cells for flower development independent of *AG*. (A) The inflorescence and phyllotaxy abnormalities in a *lsn-1* plant. (B) The *ag-1* inflorescence and phyllotaxy. (C) The inflorescence and abnormal phyllotaxy in an *ag-1 lsn-1* double mutant plant.

Map-based cloning of *LSN*

lsn-1 exhibited several interesting and novel phenotypes. First, not all flowers are abnormal. Second, whorl 1 carpels are congenitally fused in abnormal flowers. Third, acf that are subtended by filamentous or carpelloid bracts are produced in late reproductive phase. And finally, *lsn-1* exhibits phyllotaxy abnormalities. Thus, *lsn-1* is unlikely an allele of novel *AG* repressors. To identify the protein that *LSN* encodes, map-based cloning was conducted. To this end, *lsn-1 lug-2* plants in L*er* background were crossed to wild-type Columbia (Col) plants. A total of 95 F2 *lsn-1 lug-2* double mutant plants were analyzed with PCR-based molecular markers that differentiate the polymorphisms between Col and Ler (Table 2-1). *LSN* gene was mapped to BAC

T7H20 on chromosome V between markers 14K and 60K (Fig 2-10 A). Further sequencing of PCR amplified *lsn-1* and Ler genomic DNA fragments between these two markers revealed a single C to T transition in a putative homeobox gene T7H20.80 (Fig 2-10B, C) (locus: at5g02030; TAIR accession #:1005898793). The open reading frame of this gene includes four putative exons (Fig 2-10 B). The mutation was located in the second putative exon and causes an amino acid substitution from proline$_{356}$ to leucine$_{356}$ (Fig 2-10 D).



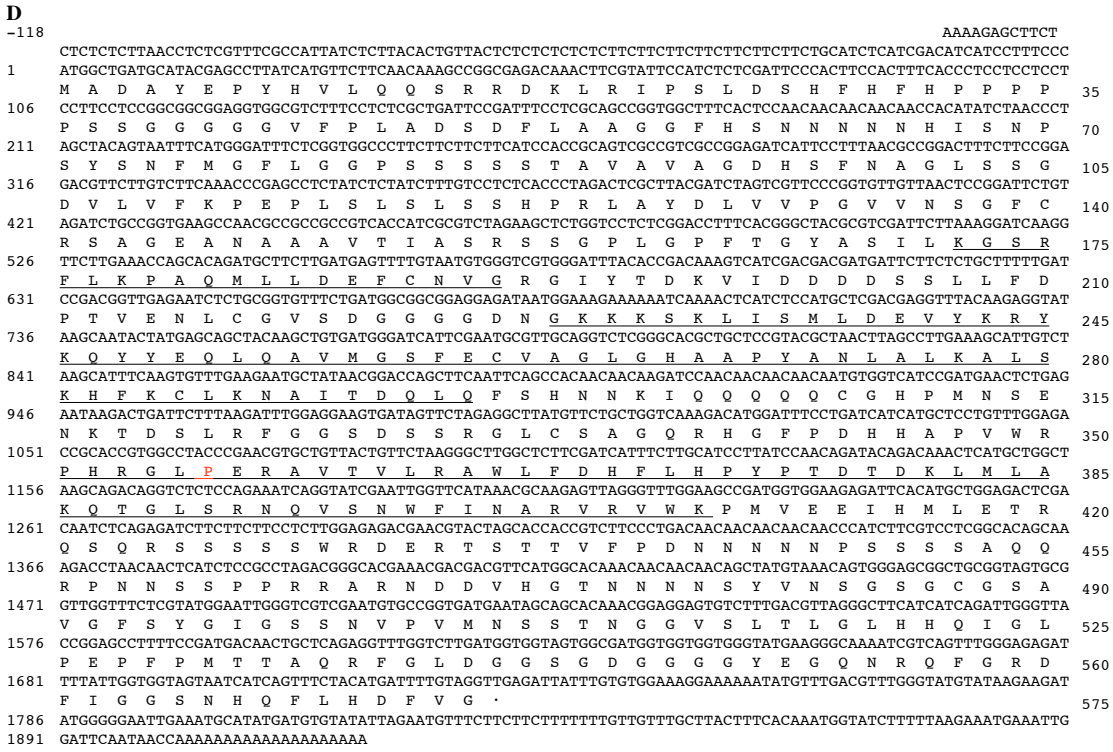


Fig 2-10. Map based cloning of *LSN*. (A) PCR-based molecular markers and number of recombinants. (B) Gene structure of putative *LSN*. Numbers indicate the DNA sequence length in base pairs. (C) The functional domains of LSN. Numbers indicate amino acid residues. Conserved domains are predicted based on homology to BELL1 protein. (D) The full-length *LSN* cDNA sequence and the translated protein sequence. Numbers on the left and right indicate nucleotide positions and amino acid residue positions, respectively. Conserved domains are underlined. Site of *lsn-1* mutation is in red. Chr, Chromosome; SKY, SKY domain; BEL, BELL domain; HD, homeodomain.

To obtain *LSN* cDNA, a flower cDNA library was screened using *LSN* exon I as the probe. Two positive clones were obtained after screening ~5.5 x 10^5 phage plaques. These two clones were identical to each other. The 3' end of the cDNA contains a poly-

A tail. This cDNA was 12 nucleotides shorter than the full-length *LSN* cDNA determined by 5'RACE. Sequence of the cDNA clone also showed that the splicing pattern of *LSN* gene was the same as the AGI annotation (Fig 2-10 D).

Genomic complementation

To further confirm that T7H20.80 is indeed *LSN*, a DNA fragment containing T7H20.80, 3426bp upstream, and 2455bp downstream was excised from BAC T7H20 using BamH I and Spe I. This fragment was cloned into pBIN binary vector (Hennegan & Danna 1998) and transformed into *lsn-1* homozygous plants via an Agrobacterium-mediated method. Kanamycin resistant T1 plants were grown at 29 ^{o}C. Six out of seventeen transgenic *lsn-1* plants showed normal phyllotaxy and flowers. T2 seeds are collected from these wild type plants. On average, approximately 3/4 of the T2 progeny (28.2±5.63 out of totally 37.7±8.91 T2 progenies from those six T1 plants) exhibited wild-type flower and normal phyllotaxy at 29 ^{o}C. The segregation appeared to follow the Mendelian ratio for dominant traits. Therefore, T7H20.80 encodes LSN.

LSN is expressed in the inflorescence and floral meristem

To explain the shoot apical and flower phenotypes, *LSN* expression in different tissues was investigated. When total RNA from different plant tissues was used in Northern hybridization, *LSN* signal could not be detected. RT-PCR analysis indicated that *LSN* was not expressed in roots, but was weakly expressed in leaves and siliques (Fig 2-11A). The expression level was elevated in stem and was the highest in inflorescences (shoot apices plus flowers up to stage 14) (Fig 2-11A).

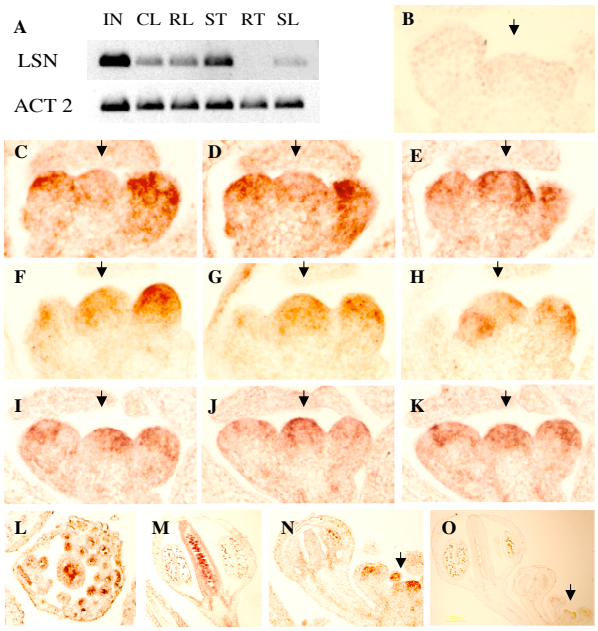


Fig 2-11. *LSN* expression profiles. (A) *LSN* RT-PCR. (B) - (O) *LSN* mRNA in situ hybridization. (B) and (O) Negative controls using sense probe. (C) - (N) Anti-sense probe. (C) - (E) Serial sections of the same inflorescence meristem. (F) - (H) Serial sections of another inflorescence meristem. (I) - (K) Serial sections of third inflorescence meristem bearing a stage 3 flower on the left and late stage 2 flower on the right. (L) A cross-section of a stage 10 flower. (M) A stage 7 flower. (N) A stage 5 flower (left) and a stage 4 flower (middle). (O) A stage 6 flower (left) and a stage 4 flower (middle). Arrows indicate inflorescence meristems. IN, inflorescence; CL, cauline leaves; RL, rosette leaves; ST, stems; RT, roots; SL, siliques.

The *LSN* expression patterns detected by *in situ* hybridization seem rather dynamic in both inflorescence and floral meristems (Fig 2-11 C to N). Amount of *LSN* transcripts often increase in the peripheral zone (Fig 2-11E, H, I). However, *LSN* hybridization signals were variable in cells. *LSN* transcription is only elevated in patches of cells in the peripheral zone of the inflorescence meristem.

LSN mRNA is highly expressed in the stage 2 flowers but gradually reduced in the central zone as the flowers grow older (Fig 11C-H). At late stage 2 and early stage

3, the *LSN* expression in central zone is obviously lower than that in the peripheral zone. The *LSN* transcripts, however, are not forming a continuous ring. Instead, as in the inflorescence meristem, the mRNA level is high in one section (Fig 2-11I, K) and is reduced in another of the same flower (Fig 2-11J). Therefore *LSN* is likely active in cells that are about to differentiate to floral organ primordial cells during early flower development. Unexpectedly, *LSN* expression is significantly reduced at the sites where sepal and petal primordia are emerging in stage 3 and later (Fig 2-11I – K, M, N). After stage 7, *LSN* transcripts are mostly seen in ovules and stamens. Ovules show strong hybridization signals (Fig 2-11L, M).

LSN directly binds to *AG* enhancer

Molecular cloning showed that LSN is a homeodomain protein, suggesting it could bind the target gene enhancer to convey its regulator functions. To test whether LSN binds to *AG* DNA, a low yield of recombinant LSN protein was produced in the form of GST-LSN from bacterial inclusion bodies. This protein ran on SDS-PAGE at approximately 98 kD, as expected for GST-LSN protein. It was also recognized by an □-GST antibody in western assays. This protein was absent if the bacteria were not induced by IPTG or when the bacteria were transformed with the control vector (Fig 2-12). GST-LSN recombinant protein was unstable when re-folded and stored. Multiple bands were often observed in Western blots due to protein degradation. Thus protein extractions were stored as inclusion bodies to slow down the decay of GST-LSN. Freshly re-folded GST-LSN proteins (within a week) were tested for DNA-binding activities.

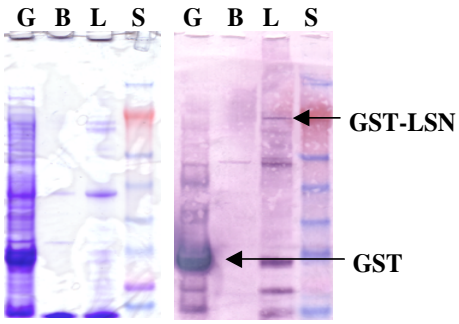


Fig 2-12. Unstable GST-LSN recombinant protein produced by BL21 *E. coli*. Left panel shows a SDS-PAGE gel stained with Coomassie blue. Right panel shows a Western blot (□-GST) of a gel duplicating the left. G, the insoluble fraction of IPTG induced pGEX-3x vector transformants; B, the insoluble fraction of pGEL (GST-LSN fusion construct) transformants in the absence of IPTG induction; L, the insoluble fraction of pGEL transformants induced with IPTG; S. protein size standards (SeeBlue-plus, Invitrogen).

Using these protein extractions, electrophoretic mobility shift assays (EMSA) were conducted using a series of radioactively labeled DNA fragments spanning the second intron of *AG*, where the *AG* enhancer resides. DNase I was added after incubating protein extracts with *AG* DNA fragments. Repeatedly, all DNA probes were digested into small pieces and migrated to the bottom of the gel except the AGb fragment that produced a retarded band indicating partial protection of AGb by the bound GST-LSN protein (Fig 2-13A, B). Bacterial and GST proteins, however, did not protect any of the DNA fragments from DNase I digestion.

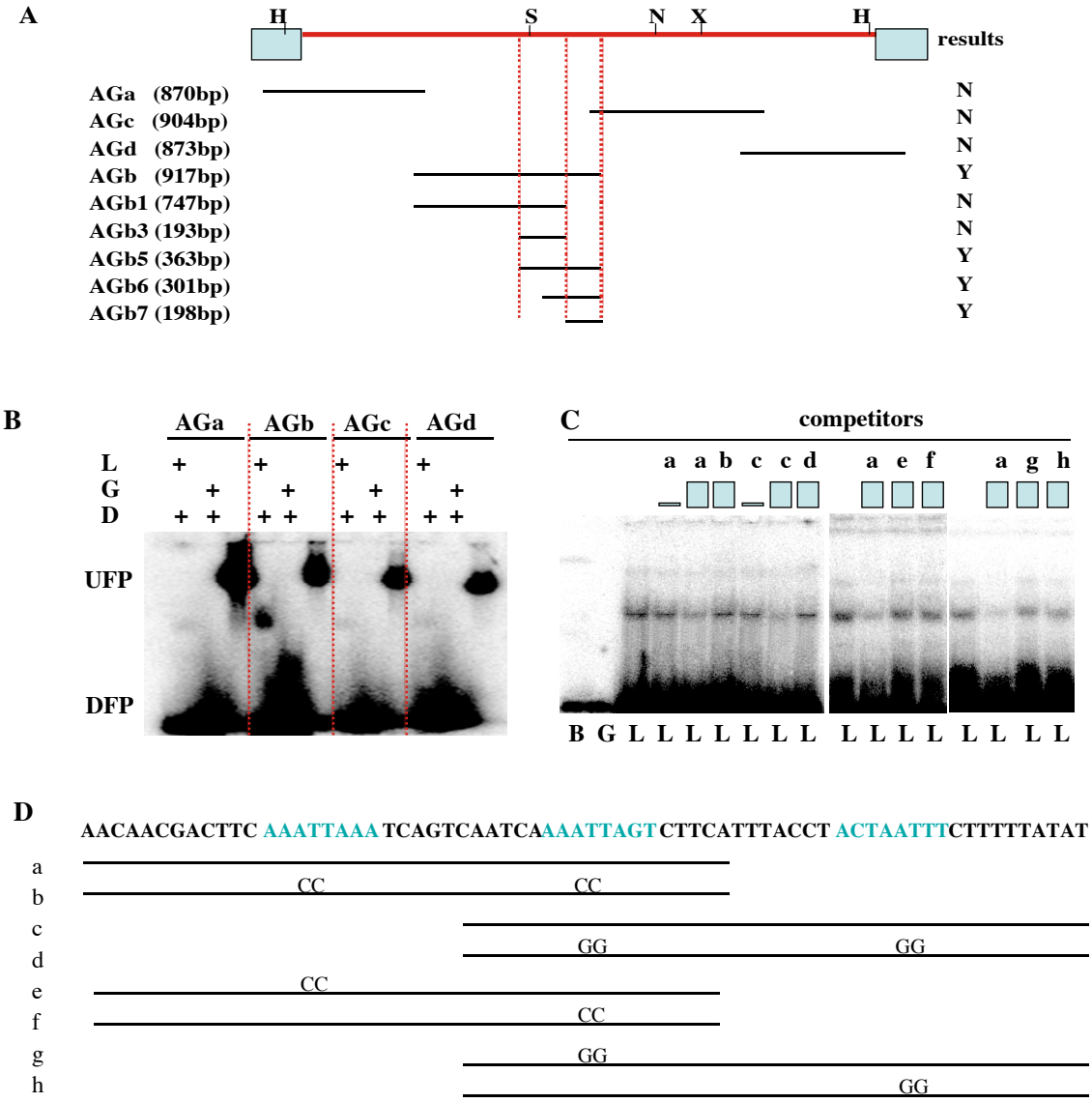


Fig 2-13. LSN directly binds to the *AG cis*-regulatory elements *in vitro*. (A) *AG* enhancer was dissected into small fragments by PCR and tested in EMSA. Results from EMSA are listed on the right. Sizes of DNA fragments are listed on the left. The red bar indicates the restriction map of *AG* second intron region (H, Hind III; S, Spe I; N, Nla IV; X, Xba I). (B) A DNase I protection assay. (C) Competition assays using cold synthetic probes shown in D. Heights of rectangles indicate relative amounts of

competitors (0.02 or 2 pmol). (D) Sequences of cold competitors used in (C). B, bacterial proteins; D, DNase I; G, GST protein; L, impure GST-LSN protein; DFP, digested free probes; UFP, un-digested free probes.

AGb was further dissected into smaller fragments by PCR and tested in EMSA. The AGb7 (198 bp) was the shortest PCR fragment whose mobility was retarded by GST-LSN protein extractions (Fig 2-13). Protein extractions from un-induced bacteria and from bacteria that expressed GST did not bind to this DNA fragment. This fragment contains three [ATTA] core sequences that are present in most of the homeodomain protein binding sites. Synthetic DNA containing two putative LSN binding sites (competitors a and c) were able to compete with AGb7 probe in binding reactions (Fig 2-13C, D). Increasing the amount of competitors from 0.02 pmol to 2 pmol almost abolished binding between GST-LSN and the radioactive AGb7 DNA. If both of the putative core sequences were mutated (competitors b and d), competitors did not compete with AGb7. Mutating a single core sequence of a competitor (competitors e, f, g, and h) significantly reduced its ability to compete with AGb7 for GST-LSN binding. Notably, however, the free probes that migrated to the bottom of the gel exhibited tails instead of condensed distinctive bands. This might indicate that the GST-LSN/DNA complex was not stable and the gel runing buffer conditions may have caused dissociations of these GST-LSN/DNA complexes.

The LSN binding sites were partially conserved in Brassicaceae

Using reporter gene analyses paired with phylogenetic footprinting and shadowing, Hong *et al*. (2003) showed that some invariable *cis*-regulatory elements of *AG* exhibited conserved functions across Brassicaceae species or even outside of Brassicaceae. Although that study demonstrated that sequence-based studies alone were insufficient for a complete identification of *cis*-regulatory elements, it is important to evaluate the conservation of newly identified *cis*-elements across related and unrelated taxa. Taking advantage of the sequence information reported by Hong et al (2003), 29 *AG* enhancer sequences in Brassicaceae and 12 orthologous sequences of non-Brassicaceae *AG* enhancer were evaluated for the conservation of LSN-binding sites using sliding-window analysis. Total of 17 out of the 29 *AG* enhancer sequences in Brassicaceae, but not in any non-Brassicaceae species examined, contained a highly conserved motif that was consisted of the two downstream LSN-binding sites that had been identified by EMSA (Fig 2-14). The two [TAAT] core sequences of homedomain protein binding sites were located at the two ends of the motif. The sequence between the two EMSA detected LSN-bing sites was also highly conserved in the 17 *AG* enhancer sequences. More than 75% sequence identity was observed between any two species. Only one of such motif was found in each of the 17 *AG* enhancer sequences. The relative positions of the motif within each sequence were also conserved. The 5' end LSN-binding site detected by EMSA was not conserved. It appeared to be unique to *Arbidopsis thaliana*.

Consensus		**AAATTAaTcTTcATTtACcTAATaattt**		**Positions**
Arabidopsis thaliana	**AATCA**	**AAATTAGTCTTCATTTACCTACTAATTT**	**CTTTTTA**	**1535**
Arabidopsis arenosa	**AATCA**	**ATATTAGTCTTCATTTACCTAATAATTT**	**TTTAATA**	**1590**
Arabidopsis lyrata	**ACTCA**	**ATATTAGTCTTTATTTACCTAATATTTT**	**TTTTATC**	**1573**
Arabidopsis pumila	**CAATC**	**AAATTAATTTTCATTTACCTAATATTTA**	**TATTTTT**	**1557**
Barbarea vulgaris	**CAGTC**	**AAATTAATCTTCATTTACCTAATAATTT**	**GTTTATA**	**1740**
Guillenia flavescens 1	**ATCAA**	**AAAATAATCTTTATTCACCTAATAATAT**	**TTTTACA**	**1539**
Erysimum cheiri	**AGTCA**	**AAATTAATCTTCATTTACCTAATAAATA**	**TTCTTTT**	**2036**
Thysanocarpus sp.	**AATCA**	**AAATTAATCTTCATTTACCTAACAACTT**	**TTTGTAG**	**1535**
Conringia orientalis	**ATCAA**	**AAATTAATCTTCATTTACCTAATTAATA**	**AATTTTT**	**1605**
Nasturtium officinale	**CAGTC**	**AAATTAATCTTCACTTACCTAATAATTT**	**TTTTATA**	**1685**
Erysimum capitatum	**AGTCA**	**AAATTAATCTTCATTTACCTAATAAATT**	**TTAATAT**	**1630**
Lepidium phlebopetalum 1	**CAATC**	**AAATTAATCTTCATTTAATCAATAATTT**	**TTTTTAA**	**1339**
Lepidium africanum	**CAATC**	**AAATTAATCTTCATTTACATAATAGTAA**	**TTTTCTC**	**1307**
Coronopus squamatus 1	**AATCG**	**AAATTACTCTTTATTTACGTAATGATAT**	**ATATTTT**	**1355**
Arabis gunnisoniana	**CAATC**	**AAATTAATTTTCATTGACCTAATATCTT**	**TTTAAAA**	**1598**
Capsella b-pastoris	**CAATC**	**AATTTAATTTTCATTGACCTAATAACAT**	**TTAAAAT**	**1613**
Capsella rubella 1	**CAATC**	**AATTTAATTTTCATTGACCTAATAACAT**	**TTAAAAA**	**1617**

Fig 2-14. The LSN-binding sites were partially conserved in some Brassicaceae species. The species names were listed on the right. The positions of the the first nucleotide (5') of the motif were desgnated as the positions of the motif within each *AG* enhancer sequence and were listed on the left. The positionaing of each nucleotide was based on Hong *et al.* (2003). Extra sequences were also shown for each motif on both sides. The motif was underlined. The variable nucleotides were in blue.

DISCUSSION

LSN homeodomain protein and its homologs

LSN belongs to the TALE (Three Amino acid Loop Extension) superclass of homeobox genes (Fig 1-3). This TALE superclass includes plant KNOX and BEL classes, animal IRO, TGIF, CUP, PBC, and MEIS calsses, and yeast M-ATYP class (see review in chapter one). *LSN* belongs to the BEL class that is uniquely found in plants. *BELL1* is the founding member of this class and plays an important role in ovule integuments development in *Arabidopsis*. In total, 12 BEL class members are

predicted in the *Arabidopsis* genome (Fig 2-15A) (AGI 2000). The overall protein similarities among these homologs vary from 14% to 43%. However, the homeodomain of these proteins are essentially identical (> 80% identity) (Fig 2-15 A). Amino acid conservation is even extended outside the homeodomain, suggesting the homeodomains may not be important for the specificities of DNA-recognition by the BEL class proteins. BEL class proteins possess two additionally conserved SKY and BELL domains (Fig 2-10C) that may involve in the interactions with other homeodomain proteins (Fig 2-10C) (Bellaoui *et al.* 2001).

Typical TALE homeobox proteins contain a 60-amino acid homeodomain consisting of three α-helices that pack around a hydrophobic core. The α-helix 3 is the most conserved among the three α-helices and directly contacts the major groove of the DNA double helix (Fig2-15B) (Bürglin 1997). The N-terminal arm of the helix 1 inserts into the adjacent minor groove and interacts with DNA via hydrogen bonding between arginine and thymine (Billeter, 1996, Gehring *et al.* 1994, Pabo and Sauer 1992). Extra amino acids, the loop extension characterized with a proline-tyrosine-proline (PYP) consensus, are accommodated between helix 1 and 2 (Bürglin 1997). In *lsn-1* mutation, a leucine (L) takes the place of proline (P) at the N-terminal arm of α-helix 1. Mutations at the same position of homeodomain proteins cause genetic diseases in human (Fig 1-3) (D'Elia et al 2001). It is highly possible that *lsn-1* alters the specificity or affinity of LSN-1 protein for DNA-binding or protein interactions, or both.

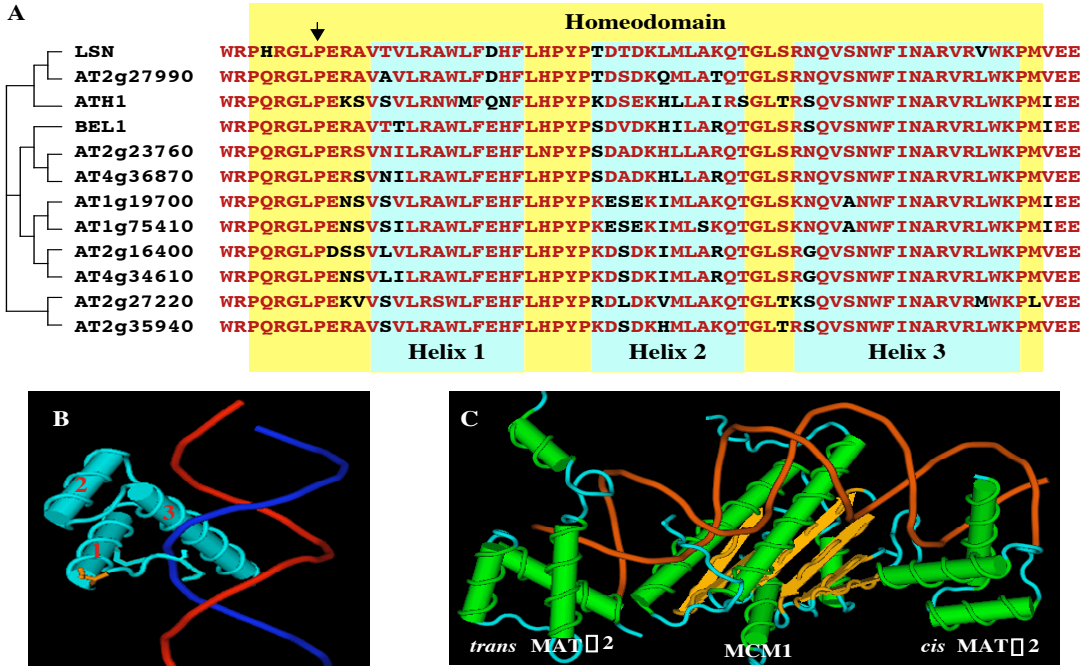


Fig 2-15. LSN homologs and DNA-binding. (A) Similarities among 12 LSN homologues in *Arabidopsis*. The dendrogram on the left is based on full-length protein sequence similarities. The homeodomain sequences are aligned on the right. Variable amino acid residues are in black letters. The arrow indicates the *lsn-1* mutation site. (B) The general configuration of homeodomain proteins when binding DNA. The orange colored position indicates predicted *lsn-1* mutation site. Numbers indicate the three □-helices. (C) Homeodomain proteins (MAT□2) may dimerize with MADS box proteins (MCM1) and homeodomain proteins (MAT□2) when binding DNA. The X-ray crystallography figures are adapted from Tan & Richmond, 1998.

TALE homeobox proteins play essential roles in regulating a wide range of biological processes. In yeast *Saccharomyces cerevisiae*, co-repressor TUP1, a

homolog of the *AG* repressor LUG, is recruited by MAT2 (M-ATYP class TALE homeodomain protein) to **a**-specific genes to convey transcription repression in haploid -cells. In contrast, MAT2 can also interact with MAT1 (homeodomain protein) to repress haploid-specific genes in diploid cells (Johnson, 1995). MAT2 cooperates with MADS box protein MCM1 to bind DNA *in vitro* (Tan & Richmond, 1998). The X-ray crystallography of MAT2/MCM1/ MCM1/DNA ternary structure provided several important observations (Fig 2-15C) (Tan & Richmond, 1998). First, protein-protein interactions are responsible for cooperative binding of MAT2 and MCM1 to DNA. This agrees with the notion that eukaryotic homeodomain proteins have low DNA-binding specificity and affinity unless they collaborate with other transcription factors. Second, DNA bending by MCM1 brings MAT2 and MCM1 close to facilitate their protein/protein interactions. Third, -hairpin at the N-terminal arms of the homeobox proteins interact with the four antiparallel -strands of the MADS box proteins via hydrogen bonds and close-packed side chains.

The binding affinity of MAT2 to its target DNA increases 50 – 500 fold in the presence of MCM1 (Keleher, *et al.* 1988, Kekeher *et al.* 1989). Based on the studies of MAT2, LSN may require partners when it binds to *AG cis*-elements. This model may partially explain difficulties, such as dissociation of the LSN/DNA complex, encountered in the LSN EMSA experiments. Similar examples exist in maize. Quantitative assays showed that the TALE homeodomain protein KNOTTED1 had a low DNA-binding affinity when acting alone but had over ten-fold higher affinity for

DNA when acting with another BEL class protein KIP (KNOTTED Interacting Protein) (Smith *et al.* 2002).

Did EMSA identify *bona-fide* LSN binding sites in plants?

The EMSA experiments showed that there are three LSN-binding sites in close proximity to each other located within the *AG* second intron. The multiple sites may suggest cooperative bindings among LSN or/and other unidentified DNA-binding proteins. Nevertheless, it will be important to test the functional relevance of these binding sites *in planta*. Previous studies using GUS reporter gene systems suggested that the specific regions of *AG* second intron containing the putative LSN-binding sites are important for *AG* repression in the shoot apex (Bomblies *et al.* 1999, Busch *et al.* 1999). The putative LSN-binding sites are located in the non-overlapping region between KB14 and KB11 (Fig 2-2). KB11, which lacks the three LSN binding sites, shows ectopic *AG* enhancer activity in inflorescence meristems while KB14, which contains the three LSN binding sites, shows normal activity. It will be interesting to mutate the KB14 enhancer at the putative LSN binding sites and examine its activity *in planta*. If the mutation causes ectopic GUS expression, the LSN binding sites identified by EMSA will then be biologically relevant.

Partial conservation of the LSN-binding motif (Fig 2-14) in some Brassicaceae species was an interesting observation. On one hand, the positions and sequences of the motif were highly conserved in the 17 out of 29 Brassicaceae species examined, but not in any of the 12 non-Brassicaceae species. The occurrence of the motif in the

Brassicaceae species could not been explained by chance alone. On the other hand, these 17 species were not distributed in any specific taxa within Brassicaceae (data not shown). Therefore, this motif is not likely evolved after speciation of Brassicaceae. Instead, there probably had been an ancestral LSN-binding motif before the evolutionary divergent point of Brassicaceae species. This ancestral motif had been lost by some species in the courses of evolution. However, this does not necessary reflect the importance of *AG* regulation by *LSN* because there are possibilities of coevolution between LSN and its binding motif. Furthermore, recognition of DNA sequences of LSN may not be crucial to its biological functions. Redundant factors of LSN or protein partners of LSN may also play important roles in determining DNA sequence recognition and binding affinity.

LSN is a novel *AG* repressor

LSN plays important roles in determining initial cell positions in the inflorescence meristem and in the repression of *AG* in flowers and in inflorescences. How *LSN* participate in the phyllotactic patterning has been discussed by Byrne *et al.* (2003) and Smith and Hake (2003). These studies showed that LSN interacts with KNAT1 and decide initial cell positions via an unknown mechanism. Because *lsn-1* mutant plants fail to repress *AG, AG* is ectopically expressed in the outer two whorls of early flowers and in the inflorescences. Homeotic transformations are observed in the organs arose from these positions. The floral homeotic phenotypes in *lsn-1* are largely similar to mutations of known flower specific *AG* repressors such as *lug, seu* and *ap2*. However, *lsn-1* exhibits at least four unique characteristics. First, not all flowers are

abnormal (see quantification in chapter three). *lsn-1* plants only produce abnormal flowers in the late reproductive phase. Temperature enhances the production of abnormal flowers. Second, unlike known mutants of *AG* repressors, such as *ap2*, *lug*, *seu*, and *clf*, the first whorl carpels of *lsn-1* flowers are more likely to be congenitally fused. Congenital fusion of first whorl organs indicates that *LSN* plays roles in organ fusion and in the formation of replum and transmitting tissues. *LSN* (also named *REPLUMLESS*) represses *SHATTERPROOF* MADS box genes (*SHP1/2*) in replum cells so that *SHP1/2* expressions are constrained in a narrow stripe of cells forming the valve margin precisely at the valve/replum boundary (Roeder *et al.* 2003). Third, a filament or a carpelloid bract subtends most of abnormal flowers. How does *LSN* repress filament/bract initiation is yet to be elucidated. Finally and most strikingly, ectopic expression of *AG* in the shoot apex caused partial homeotic transformation of the inflorescence meristems to floral meristems. A congenitally fused carpelloid structure (or acf) forms at the shoot apex at the time when a *lsn-1* plant starts to produce abnormal flowers. An important question is whether an acf terminates a shoot. Different from *terminal flower1*, the acf does not terminate shoot from producing more abnormal flowers. Quantitative assay (chapter three) showed that *lsn-1* and wild-type plants produce the same total number (normal + abnormal flowers) of flower per shoot. Variable number of abnormal flowers may be produced per shoot. If acf terminates a shoot, acf should not be observed in all samples but only in samples that were collected when the shoot is terminating. However, acf were observed in all samples (both light microscopy and SEM) independent of sampling time. Therefore, although the molecular mechanisms are not understood, ectopic *AG* expression in the inflorescence

meristem is sufficient to initiate carpelloid structures at the shoot apex, but not sufficient to terminate the shoot, as it is in floral meristem. Lohmann *et al.* (2001) predicted that there is an unknown factor(s) that facilitates *AG* termination of the floral meristem by repressing *WUS* activities in stage 6 flowers. This unknown factor is probably specific to floral meristem and is absent in inflorescence meristem.

Activation barrier model

Unlike animals in which body parts are formed during embryogenesis or puparium, plant body parts are continuously developed during postembryonic growth. The above-ground parts are developed from cells provided by a group of pluripotencial cells located in the SAM. In flowering plants, such as *Arabidopsis*, flower organs are derived from the floral meristem (Fig 1-2). However, most molecular genetic studies of floral organogenesis in the past, such as those described in the ABCE model, isolated organs formations from their temporal developmental histories and emphasized only the spatial activities of organ identity genes. The studies described in this dissertation provide evidence for how the developmental histories determine the cell types in floral organs.

Plant cells do not slide or slip relative to one another. However, the descendant cells proliferated from the meristems do not uphold their positions relative to the SAM. Cell divisions not only allow replenishing the meristem itself but also provide new cells to the peripheral zone and rib meristem. Continued cell division in the rib meristem and peripheral zone result in the upward movement of meristem and lateral expansion of

stem diameter (Meyerowitz 1997, Steeves & Sussex 1989). During this process, cells are not simply "moving" away from the centripetal axis, but also maturing and differentiating to their destinations. How transcriptional regulatory machineries alter gene expressions during development of pluripotent cells to specific destined cells is of basic interest to developmental biologists.

In reproductive shoots, *AG* is competent for activation because the activity of trxG gene *ATX1* overwhelms that of PcG gene CLF (Alvarez-Venegas *et al.* 2003, Goodrich *et al.* 1997). In wild-type plants, however *WUS* does not activate *AG* in inflorescence meristems and stage 1-2 flowers (Lohmann *et al.* 2001). Mutating *LSN* causes ectopic expression of *AG* in the inflorescence meristem and thus, the production of apical carpelloid flowers (Fig 2-6). Therefore, *LSN*-associated machinery re-initiates *AG* repression state in inflorescence meristems, resuming the role of CLF-repression complex and acting antagonistic to the ATX1 activation complex.

LSN is expressed in the peripheral zone of floral meristem and its expression peaked in the cells that are about to be differentiated into sepal and stamen primordia (Fig 2-6, Fig 2-16). It is interesting to observe that *LSN* expression is significantly reduced in the sepal primordia of stage 3 flowers. However, mutating *LSN* cause strong ectopic activation of *AG* in the outer two whorls of flowers, leading to abnormalities in the very beginning of sepal organogenesis (Fig 2-6A, B). Therefore, unless the LSN protein traffics among cells, repression of *AG* in the outer two whorls of stage 3 flowers is independent upon *LSN* transcription in sepals and stamens, but is dependent of *LSN*

activities in the ancestral cells in stage 1 and 2 floral primordia. In other words, the cell developmental history determines the gene activation states in sepal primordial cells. The maintenance of *AG* repression in the sepal and stamen, therefore, does not require the presence of LSN mRNA.

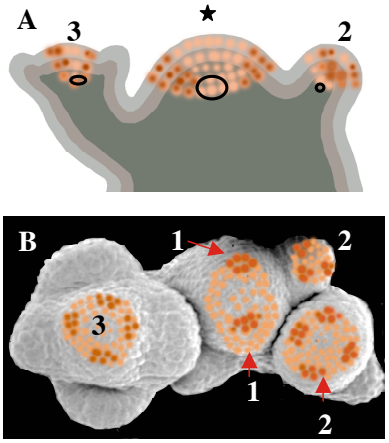


Fig 2-16. Schematic of *LSN* expression domains. (A) longitudinal view of inflorescence and floral meristems. (B) Overhead view of inflorescence and floral meristems. Artificial color intensities correlate with LSN expression levels. Numbers indicate flower stages. Asterisk indicates inflorescence meristem.

Recent advances in studies of transcriptional gene regulations have shown that repression initiation machineries in the progenitor cells may be different from the repression maintenance machineries in the descendent cells. An enhancer cannot be silenced by the maintenance machinery if the enhancer DNA has not experienced the initiation of repression (Franco *et al.* 1997, Kelly *et al.* 2003). These studies demonstrated that tissue-specific activities (regionalization) of *Mlc3f* enhancer, a crucial factor for heart organogenesis in mice, are regulated by chromatin modifying proteins. Regionalized *Mlc3f::lacZ* reporter gene activities can be established if the reporter gene is introduced into pluripotential cells before embryonic days (E) 8 to 10, when endogenous *Mlc3f* gene expression pattern is established. However, if the reporter gene

construct is introduced into cells after embryonic days (E) 10, the transgenic *Mlc3f* enhancer is activated ubiquitously, despite the fact that the endogenous *Mlc3f* is regionalized via differential DNA methylation and histone acetylation patterns.

Similar to the *Mlc3f* enhancer that needs to experience repression initiation before (E)10, *AG* enhancer may needs to experience initiation of repression by *LSN* in the floral meristem peripheral zone at stage 1-2. This imprint is maintained in sepals of stage 3 flowers. The spatial activities of *LSN* in peripheral zone cells may thus contribute to the temporal effects in the destined cells of cell lineages. The similar *LSN* expression patterns in inflorescence and floral meristems suggest a spatial barrier at which *AG* is repressed. I name the positions of meristem cells that contain elevated *LSN* expression, collectively, "activation barriers" to describe *AG* repression initiation by *LSN* at these locations.

It is necessary to mention that LSN is insufficient to repress *AG* in the inner two whorls of flowers at stage 3. The reason is that at least two synergistic activators, LFY and WUS, are acting in the floral meristem central zone to counter *LSN* repression and *LSN* expression is reduced in the central zone of stage 3 floral meristems. Therefore, activation machinery overcomes repression in the center of stage 3 floral meristems. Furthermore, more *AG* activator may be present in meristems as is predicted by studies in transgenic plants (Hong *et al.* 2003). In this study, an *AG* enhancer containing a mutated MADS box protein binding site, namely CArG box 1 located 3' of the *AG* second intron, caused ectopic expression of the reporter gene in inflorescence meristem

where *LFY* is not normally expressed. This ectopic activation of the *AG* enhancer is not affected by the absence of LFY in *lfy-12* plants. However, mutating the putative LFY binding site in this *AG* enhancer abolished the ectopic *AG* in inflorescences. These data indicate the existence of unknown *AG* activators in the inflorescence, which may require LFY binding sites but not LFY proteins.

To prove the activation barrier model, it is necessary first, to prove that LSN protein is only distributed in the pre-organ and floral primordial cells but not in sepal and stamen primordia. To directly test this, LSN protein localization needs to be determined using a LSN specific antibody or observing epitope-tagged LSN *in planta*. Unfortunately, an attempt to raise LSN specific antibody has failed. Second, it is necessary to show that *LSN* can initiate *AG* repression in plant cells, especially in cells that the CLF repression complex is not functioning, such as in leave cells of *clf-2*, in which *AG* mRNA is detectable by Northern blot (Goodrich et al, 1997). An inducible *LSN* expression construct can be transformed into *clf-2 lsn-1* plants. *AG* expression level in leaves can be examined before, during, and after induction of *LSN*. If *LSN* plays a role in repression initiation but not in maintenance, inducible *AG* repression would not be maintained when *LSN* induction is removed.

The next logical step is to examine the molecular mechanism by which *LSN* represses *AG*. It is commonly observed that transcription repression in eukaryotic cells is caused by epigenetic modifications of chromatin structures of the target enhancers. *LSN* may act in a similar manner. That is, LSN probably recruits chromatin-modifying

factors to the *AG* enhancer region, leading to re-establishment of epigenetic repression state specific to *AG* before primordia initiation in the peripheral zone. This repression state may be inherited to the descendent cells in the floral organs. A good candidate for LSN associated chromatin modifying factor is AtGCN5 (histone acetyltransferase). AtGCN5 acts in both inflorescence meristem and flowers. Mutating *AtGCN5* causes ectopic expression of *AG* in the inflorescence meristem and thus, growth of fused carpels at the apex (Bertrand et al 2003).

It is also very important to identify the maintenance complex that passes on the *LSN*-initiated repression state to the descendent cells. Equally important are how the maintenance complex is subsequently sequestered to the *AG cis*-elements and how these *cis*-elements are differ from the LSN-binding sites in *AG* intron. In plants, the only gene for which the transcription regulation has been investigated in similar depth comparable to *AG* is *FLC* (Gendall *et al.* 2001, Sung & Amasino 2004). At present, it is known that the repression of *FLC* is initiated by VIN3, a homeodomain protein containing a PHD domain, and a nonspecific DNA-binding protein VRN1. The repression of *FLC* is maintained by PcG protein VRN2. These type of studies will shed lights on how genes are regulated and how gene regulation affects cell development in plants and other multicellular eukaryotic systems.

Chapter three

INTERACTIONS BETWEEN *LSN* AND *AG* REPRESSORS

ABSTRACT

Molecular and genetic analysis in chapter two suggested that LSN might recruite cofactors to *AG* enhancer to repress *AG*. To understand the molecular and genetic interactions among flower specific *AG* repressors, genetic and molecular interactions amoung *LSN, LUG, SEU, AP1* and *AP2* were investigated or reviewed in this chapter. Data suggested that LSN may act upstream of a putative LUG/SEU/AP1 complex, which may involved in maintenance of *AG* repression organ primordia. Further tests were proposed to examine the hypothesis that LSN and the putative LUG/SEU/AP1 complex participate *AG* repression initiation and maintenance, respectively.

INTRODUCTION

The phase transition from vegetative development to flowering in *Arabidopsis* is marked by two important events. First, chromatin remodeling by the trxG gene *ATX1* switches the epigenetic state of floral organ identity genes from repressive to active (Alvarez-Venegas *et al.* 2003). Second, the floral meristem identity gene *LFY* is activated. The expression levels of A, B, C and E class genes are all reduced in *atx1-1* mutant. Interestingly, both wild-type and severe mutant floral organs are present in the same flower of *atx1-1* plants. This phenotype suggests both normal and abnormal epigenetic regulations occur in different cell lineages of the same genetic background in *atx1*plants.

In wild-type plants, *ATX1* is expressed in the inflorescence meristem and peaks in flower primordial, providing transcriptional competence for *AG* enhancer in both inflorescence meristem and floral primordia. However, elevated *LSN* expression in these regions resumes *AG* repression so that *AG* transcription is not initiated until the flowers develop to stage 3. Furthermore, *LSN* is required for *AG* repression in the floral meristem peripheral zone in stage 1-2 flowers. This repression state is probably inherited to the descendent cells in the organs arising in the outer two whorls, where repression could be maintained even in the absence of LSN in these tissues. *LSN* activity alone is necessary but not sufficient to repress *AG* in the outer two whorls of flowers. The reason is that several genes have been shown to be essential to repress *AG* in the outer two whorls of flowers.

Flower specific *AG* repressor *LEUNIG (LUG)*

LUG is expressed in most parts of the plant, and is highly expressed in the inflorescence meristems and early stage flowers (Conner & Liu 2000). In *lug* mutants, *AG* is precociously expressed in stage 2 flower primordia and ectopically expressed in the sepal and petal primordia. As reviewed in chapter three, an *AG* enhancer region responds to the presence and absence of *LUG* in plants (Deyholos & Sieburth 2000). Therefore *LUG* directly or indirectly acts upon *AG* enhancer to exert its repressor activities.

LUG is a glutamine-rich (Q-rich) protein with seven WD repeats at the C-terminus and a LUFS domain at the N-terminus (Conner & Liu 2000). The protein is localized in the nucleus and share structural motifs (the Q-rich domain and WD repeats) with transcriptional corepressors *Groucho* (Gro) in *Drosophila, transducin-like enhancer of split* (*TLE*) in humans, and *TUP1* in yeast (collectively GroTLE family proteins) (Chen & Courey, 2000)**.** GroTLE family proteins repress a number of genes via their association with DNA-binding transcription factors (Dubnicoff *et al.* 1997, Keleher *et al.* 1992, Paroush *et al.* 1994). Once recruited by the DNA-binding factors, GroTLE proteins interact with chromatin modifying factors such as histone deacetylases, which lead to epigenetic silencing of target genes (Calvo *et al.* 2001, Chen *et al.* 1999, Winnier *et al.* 1999). Importantly, GroTLE family proteins, such as *Gro*, are known to be able to convert activators, such as *Dorsal* and *dTcf,* into repressors in *Drosophila* (Chen & Courey 2000). It is not the DNA-binding specificity but the type of proteins recruited by the transcription factors that determines the transcription state of the target genes.

Flower specific *AG* repressor *SEUSS (SEU)*

seu is a genetic enhancer of *lug* in *AG* regulation (Franks *et al.* 2002). Precocious and ectopic expressions of *AG* in *seu* early flower primordia and in the outer two whorls of flowers cause partial homeotic transformations of floral organ identities and slight reductions of floral organ numbers. *seu lug* double mutant plants exhibit drastically enhanced phenotype, with a greatly enhanced reduction of floral organ numbers and more complete homeotic transformations. Similar to *LUG*, *SEU* is

expressed in leaves, and its expression peaks in flowers. Protein-protein interactions between LUG and SEU have been shown in yeast and *in vitro* pull-down assays (Surendrarao, Sridhar & Liu unpublished data).

SEU contains two Q-rich domains at the N- and C-terminal arm of a highly conserved dimerization domain (DD). The DD domain is found in LIM-domain-binding proteins (Ldb) in animals (Franks *et al.* 2002, Matthews & Visvader 2003). Unlike Ldb, SEU does not contain the LIM-interaction domain (LID) that is present in all Ldb proteins. Ldb family proteins act as multi-adaptor proteins that mediate interactions between different classes of transcription factors and their co-regulators to regulate a wide range of biological processes in different organisms (Matthews & Visvader 2003). Ldb proteins dimerize at the DD to form a Y shaped structure along with the two protruding LIDs. Each LID interacts with a LIM-homeodomain protein to form a tetrameric complex or interacts with LIM-homeodomain dimers. As a consequence of these protein-protein interactions, Lbd dimers bring distal *cis*-elements recognized by transcription factors into close proximity and exert their biological functions. Depending on the recruited co-regulators, the target genes may either be repressed or activated. SEU does not contain a LIM binding domain but, instead, contains two different Q-rich domains. Whether SEU acts similarly to Ldb as a molecular adaptor to mediate and stabilize DNA-binding complexes is unknown. However, genetic and molecular interactions between *SEU* and *LUG* led to a model in which SEU mediates the interaction between LUG and a unknown DNA-binding factors to repress *AG* expression (Franks *et al.* 2001).

Flower specific *AG* repressor *APETALA2 (AP2)*

Although both LUG and SEU possess protein-protein interaction domains, they do not contain any DNA-binding domains. Another *AG* repressor *AP2*, however, encodes a putative transcription factor with two 70-amino acid domains called AP2 domains that are responsible for DNA-binding specificity and affinity (Nole-Wilson & Krizek 2000). Each of the AP2 domains is folded into a DNA recognition structure consisting of a three-stranded β-sheet packed against an α-helix. Residues in the β-sheet are primarily responsible for DNA recognition (Allen *et al.* 1998). One AP2 domain is proposed to contact the 5' half of the binding site on the DNA template and the other AP2 domain contacts the 3' half of the binding site (Nole-Wilson & Krizek 2000).

In *ap2* mutants, floral organs in the outer two whorls are homeotically transformed due to ectopically expressed *AG* (Bowman *et al.*1991, Drews *et al.* 1991). *AP2* seemingly acts in a *LFY*-dependent manner as the presence or absence of LFY binding sites in the *AG* enhancer significantly affects the ability of *AG* enhancer to respond to *ap2* mutations (Bomblies *et al* 1999, Deyholos & Sieburth 2000). However, direct binding of AP2 to *AG* enhancer has not yet been established.

Interestingly, *AP2* mRNA is distributed in all four whorls of wild-type flowers (Jofuku *et al.* 1994). Two recent studies suggest that *AP2* function is not regulated at transcription, but at the translation level (Aukerman & Sakai 2003, Chen 2003). The

Arabidopsis microRNA miRNA172 is highly complementary to *AP2* mRNA and inhibits the translation of *AP2*-like genes including AP2. Elevated and ectopic miRNA172 expression resulted in a loss of *AP2* function in all four whorls, and leading to *ap2*-like floral phenotypes. Therefore, whorl 3- and 4-specific expression of miRNA172 may inhibit *AP2* translation in whorl 3-4 and result in AP2 production in whorl 1 and 2. Interestingly, *HEN1* (*HUA EHNANCER1*), a RNA helicase, which is involved in microRNA processing, is required for mi172 accumulation. *hen1* enhances *hua1* (encoding a single stranded nucleic acid-binding protein) and *hua2* (encoding a novel transcription activator), which in turn enhance *ag* (reviewed by Jack 2002). The whorl 3 stamens are transformed to sepals in *hen1 hua1 hua2* triple mutants, indicating a loss of B and C class gene activities and the expanding of A class function to whorl 3, a phenotype similar to a loss of *AG* (Jack 2002). Therefore, *AP2* and *AG* may form a negative regulatory loop in which *HEN1*, *HUA1*, *HUA2*, and mi172 play important roles in determining regionalization *AG* and *AP2* activities.

Flora meristem and floral organ identity gene *APETALA1 (AP1)*

MADS box gene *AP1* plays important roles in determining floral meristem identity and whorl 1-2 floral organ identities (Bowman *et al.* 1989, Bowman *et al.* 1991, Mandel *et al.* 1992, Huala & Sussex 1992, Weigel *et al*. 1992). *AP1* and *LFY* interact synergistically to promote floral meristem identity and activate B,C class genes in stage 3 flowers (Weigel & Meyerowitz 1993, Weigel & Nilsson 1995, Busch *et al*. 1999). In *ap1 lfy* double mutant, inflorescence meristems produce lateral shoots instead of flowers. These data may have given an impression that *AP1* is simply an *AG* activator.

However, it has long been observed that *AP1* may also play a role in *AG* repression in the outer two whorls of flowers. For example, *ap1* enhances the defects of *lug* in *AG* repression. Therefore, *AP1* may serve as either a positive or a negative regulator of *AG,* perhaps depending on its co-regulator.

AP1 is activated in the entire floral meristem at floral stage 1 and the mRNA is restricted to the outer two whorls of flowers starting at stage 3 (Gustafson-Brown *et al.* 1994, Wagner *et al.* 1999). AP1 is, therefore, a good candidate for the outer two whorl-specific regulator of *AG*, perhaps by interacting with components of the repression complex. Furthermore, yeast GroTEL family co-repressor TUP1 is known to be recruited by a TALE homeodomain protein MAT□2 and a MADS box protein MCM1 in repression of **a**-specific genes in □-cells (Johnson, 1995, Tan & Richmond, 1998). Therefore, AP1 may recognize and bind to *AG* enhancer and recruit the putative LUG/SUE co-repressors to *AG* enhancer in cooperation with other transcription factors such as LSN.

In an attempt to understand the genetic and molecular mechanisms employed by *LSN* in the negative regulation of *AG*, this chapter studies the authenticity of *lsn-1* phenotypes, and the interactions between *LSN* and several *AG* repressors. Additional *lsn* alleles were isolated using reverse-genetic approaches. To understand the molecular and genetic interactions among flower specific *AG* repressors, genetic and molecular interactions amoung *LSN, LUG, SEU, AP1* and *AP2* were investigated or reviewed in this chapter. Based upon the results discussed in this dissertation, I propose that a

repression maintenance complex exists in flowers to repress *AG* expression in the outer two whorls. Alternative models are also proposed to explain *AG* repression during early flower organogenesis. The effects of temperature on different *lsn* alleles are discussed.

MATERIAL AND METHODS

(See chapter five)

RESULTS

Multiple *lsn* alleles exhibited similar phenotypes

lsn-1 contains an amino acid substitution (P_{356} to L_{356}) at the N-terminal arm of the homeodomain. It is reasonable to suspect that LSN-1 protein may be folded into a neomorphic protein due to the drastic difference between the side chains of proline and leucine. To determine whether *lsn-1* phenotype reflects the true functions of *LSN*, two additional *lsn* alleles were isolated using reverse genetic approaches. *lsn-2* is an EMS induced missense mutant isolated by TILLING (Targeting Induced Local Lesions IN Genomes) developed in Dr. Steven Henikoff's laboratory (University of Washington, Seattle) (McCallum CM 2000a, McCallum CM2000b). *lsn-2* is located in the □-helix I of the homeodomain (R_{364}->L_{364}) (Fig 3-1A). A third allele (*lsn-3*) is caused by a Ds transponson insertion generated in Dr. Robert Martienssen's laboratory (Cold Spring Harbor laboratory, Woodbury) (Sundaresan et al 1995). The Ds transposon is inserted 2 bp upstream of the ATG translation initiation codon. RT-PCR anaysis showed that *lsn-3* plants did not produce any *LSN* mRNA (Fig 3-1B) and thus is a null allele.

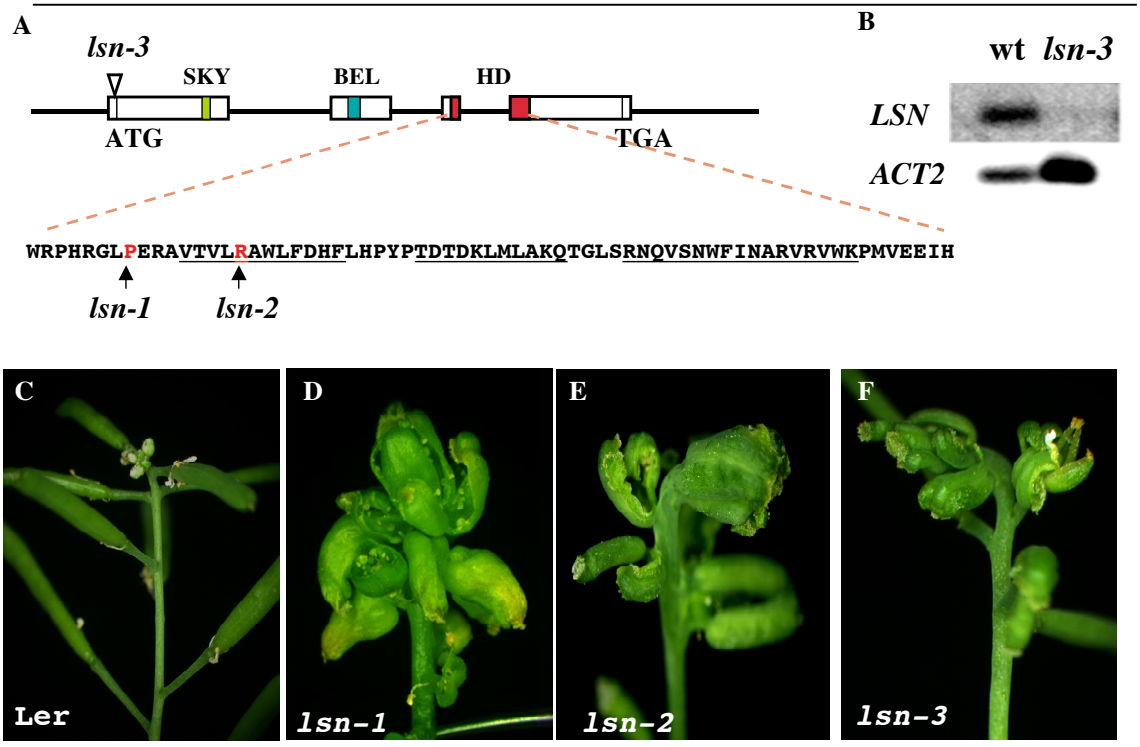


Fig 3-1. Analyses of three *lsn* alleles. (A) LSN gene structure and the positions of *lsn-1, lsn-2,* and *lsn-3*. Red letters indicate the sites of amino acid substitutes. -helices are underlined. Colored domains (SKY, BEL, and HD) are conserved in BELL type proteins. (B) RT-PCR of *LSN* and *ACT2* mRNA in wild-type (wt) and *lsn-3* inflorescences. (C) A wild-type (L*er*) shoot at late reproductive phase. (D) A shoot of *lsn-1* bearing tcf. (E) A shoot of *lsn-2* bearing tcf. (F) A shoot of *lsn-3* bearing tcf. Plants were grown at 29 ^{o}C. ACT2, *ACTIN2*.

These three alleles exhibit the same phyllotaxy abnormality. More importantly, carpelloid flowers (collectively tcf as described in chapter 3 (Fig3-5 Fig 3-6))were produced in both *lsn-2* and *lsn-3* after the production of normal flowers in the early reproductive phase (Fig 3-1 C-F). The similar phenotypes in *lsn-1* and *lsn-2* missense

alleles and, especially, in *lsn-3* null allele indicate that *lsn-1* is a loss-of-function allele, rather than a neomorphic allele. The *lsn-1* phenotypes described in chapter three indicated the true function of *LSN*.

Complementation tests

As discussed in chapter two, the tcf phenotype is only exhibited in a portion of *lsn* mutant plants. In plants that exhibited tcf, only flowers produced during the late reproductive phase showed carpelloid floral organs. Therefore, it is necessary to quantify the phenotypic severities in each *lsn* allele. In addition, *lsn-1* and *lsn-3* were isolated in L*er* background while *lsn*-2 was induced in Col background. It is important to investigate whether the ecotype differences contribute to the different phenotype severity among three *lsn* alleles. Finally, *lsn-2* was induced by a high dosage of EMS, which may introduce background mutations contributing to the phenotypic characteristics of *lsn-2*. For these reasons, trans-heterozygous plants *lsn-1/lsn-2*, *lsn-1/lsn-3*, and *lsn-2/lsn-3* were constructed by genetic crosses. These plants were grown under high (29 ^{o}C) and low (20 ^{o}C) temperatures to quantify the effect of temperature on each allele. The percentage of tcf bearing plants in each genotype (%tcf plts) and the averaged percentage carpelloid flowers produced by each primary shoot (%cf/sht) were examined (Fig 3-2).

High temperature reduced the total number of flowers produced by the primary shoot in wild-type and various lsn mutant plants tested (Table 3-1). But the total numbers of flowers per shoot at the time the shoot terminated were approximately the

same in different genotypes when the plants were grown under the same temperature (Table 3-1). *lsn-1*/+ heterozygous plants were phenotypically wild-type, indicating that the *lsn-1* mutation is recessive to the wild-type allele (Fig 3-2). In trans-heterozygotes *lsn-1/lsn-2*, *lsn-1/lsn-3*, and *lsn-2/lsn-3*, both phyllotaxy and tcf phenotypes were present. All mutant plants produced wild-type flowers in the early reproductive phase. In the late reproductive phase, some mutant plants produced tcf and some continued to produce wild-type flowers. Considerable variations among different alleles were observed for both %tcf plts and %tcf/sht. However, plants that exhibited relatively high %tcf plts always exhibited relatively high %tcf/sht. Thus, %tcf plts seemed positively correlated with %tcf/sht under different temperatures (Fig 3-2). Hence, both parameters reflected the severity (degree of penetrance) of *lsn* alleles.

Table 3-1. Total number of flowers produced by the primary shoots.

		lsn-1	*lsn-2*	*lsn-3*	*lsn-1 /lsn-2*	*lsn-1 /lsn-3*	*lsn-2 /lsn-3*	*lsn-1 /+*	*Ler*
29°C	m	25.6	30.9	36.9	30.3	32.6	32.1	33.3	27.9
	sd	5.85	8.68	6.52	6.97	5.12	4.55	6.29	3.76
	n	22	31	23	29	28	22	22	22
20°C	m	44.0	47.6	51.3	46.8	50.8	47.2	51.2	44.0
	sd	5.97	7.61	5.64	6.44	7.44	5.71	6.41	5.73
	n	25	28	25	27	25	26	17	24

m, mean; sd, standard deviation; n; number of plants.

As shown in Fig 3-2, in homozygous plants, *lsn-1* plants exhibited the most severe phenotype while *lsn-3* plants exhibited the weakest phenotype. *lsn-1/lsn-2* exhibited a more severer phenotype than *lsn-1/lsn-3*. This indicated that single copy of

lsn-2 was stronger than single copy *lsn-3,* in agreement with the degree of severity observed in the homozygous plants. Similarly, single copy of *lsn-1* is relatively stronger that single copy *lsn-2*. Ecotype background seemed to not affect both parameters tested because *lsn-2,* a Col plant, was not always stronger or weaker than the other two mutants in L*er* background. The *lsn-2* phenotype was not complemented by both *lsn-1* and *lsn-3*, indicating that the observed stronger *lsn-2* phenotype was caused by a mutation in *LSN* locus rather than unknown background mutations elsewhere.

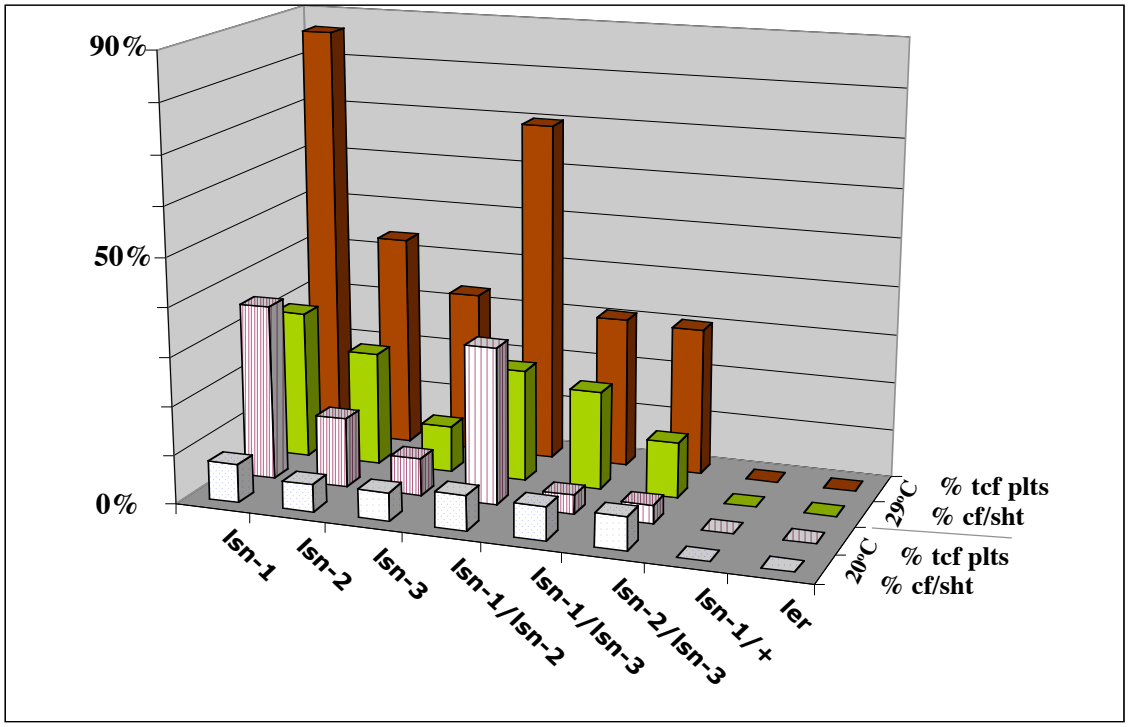


Fig 3-2. Severity of *lsn* phenotypes. Plants were grown under high (29 °C) and low (20 °C) temperatures. %tcf plts, percentage of plants bearing tcf; %cf/sht, the percentage of carpelloid flowers out of total flowers produced per primary shoot averaged for number of plants scored as in Table 3-1.

Increased temperature enhanced the tcf severity in all *lsn* genotypes. However the relative severities among the three *lsn* alleles (*lsn-1* > *lsn-2* > *lsn-3*) appeared not altered by the increased temperature. Interestingly, the responses to higher temperature are not limited to missense alleles *lsn-1* and *lsn-2*. The null allele *lsn-3* also showed a more severe tcf at high temperature than at the low temperature. This suggests that the temperature is not enhancing the tcf in an allele-specific manner but in a gene specific-manner. Therefore, *LSN* is probably regulating a temperature sensitive process.

lsn interacts with *lug*

To understand the genetic basis underlying *AG*-repression, *lsn-1* and *lsn-3* were crossed to *lug, seu,* and *ag* mutants to observe double and triple mutant phenotypes. Floral and inflorescence morphologies and tcf quantifications were compared among *lsn* double and triple mutant plants at normal growth temperature (20 ^{o}C).

In the early reproductive phase, *lug lsn* double mutants showed similar flower phenotypes to *lug* single mutant at 20 ^{o}C (Table 3-2). At the late reproductive phase *lsn lug* flowers exhibited reduced number of organs in whorl 1-4. Sepals are completely transformed into carpels evidenced by the ectopic ovules and stigmatic tissue at the tips of whorl 1 organs (Table 3-2, Fig 3-3). Unlike *lug* flowers, the first whorl organs of *lsn lug* are often fused to form an ectopic gynoecium and whorl 4 organs are either reduced or absent in (Table 3-2, Fig 3-3). Organ numbers in whorls 2-3 were drastically reduced or even absent in *lsn lug* flowers. *lsn* enhanced both weak (*lug-8*) and strong (*lug-3*) alleles (Fig 3-3).

Table 3-2. *lsn* enhances *lug* flower phenotypes at 20 °C.

Genotypes		**Flower organ numbers** (mean ± standard deviation) (n = 20)			
		Whorl 1	Whorl 2	Whorl 3	Whorl 4
lug-3		3.9 ± 0.37	2.0 ± 1.41	2.9 ± 0.97	2.0 ± 0.00
lug-3 lsn-1	Early	3.7 ± 0.29	2.1 ± 1.29	3.1 ± 0.58	2.0 ± 0
	Late	2.6 ± 0.81	0.0 ± 0.00	0.5 ± 0.12	0.7 ± 0.32

Individual flowers are collected and organ numbers are counted under a dissecting microscope. The first 5 flowers are considered early flowers. Late flowers are taken after the first 15 flowers on the primary shoot.

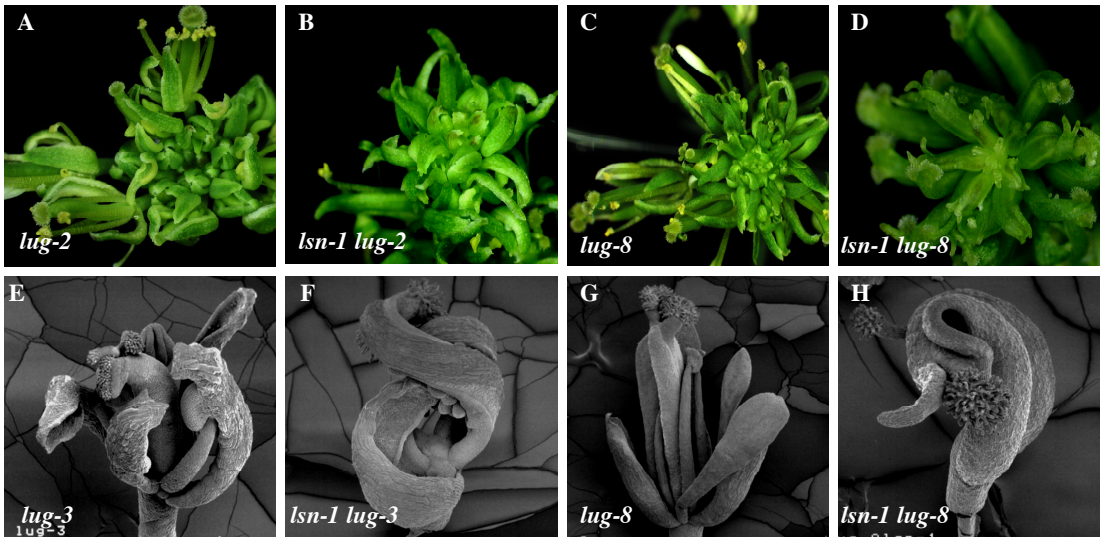


Fig 3-3. *lsn* enhanced *lug* floral phenotypes at 20 °C. (A) and (C) Inflorescences of *lug*. (B) and (D) Inflorescences of *lsn lug*. (E) and (G) SEM of flowers of *lug*. (F) and (H) SEM of *lsn lug* flowers. Samples in E-H were taken after production of first 15 flowers.

Similar to *lsn* tcf phenotypes, abnormalities of *lsn lug* started in the early stage of flowers and are manifest in late flower stages (Fig 3-4A, B). Organ initiation at the

inflorescence meristems was disorganized and the inflorescence meristems were partially transformed into floral meristem producing apical carpelloid flowers (acf). Carpelloid flowers are continuously produced after the production of the acf in the inflorescence meristem of *lug lsn*. These first whorl carpels may break at the late stages, exposing interio carpelloid organs.

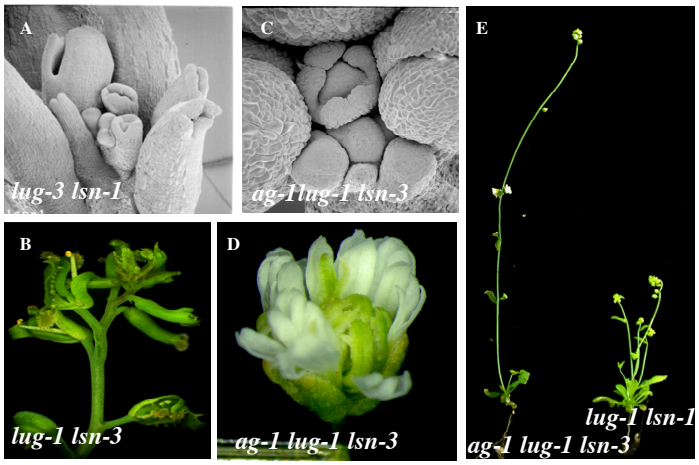


Fig 3-4. Ectopic *AG* expression mediates *lsn lug* flower phenotypes. (A) and (B) Inflorescences of *lug lsn* double mutants. (C) An inflorescence of *lug-1 lsn-1 ag-1* mutant plant. (D) A *lug-1 lsn-1 ag-1* flower. (E) Phyllotaxy of *lug-1 lsn-1 ag-1* and *lug-1 lsn-1*plants.

As expected, the *lug lsn* tcf morphology was suppressed and the organ identity defects were corrected when *AG* was removed from *lug lsn* plants by constructing *lug lsn ag* triple mutant plants (Fig 3-4C, D). The inflorescence meristem morphology of this *lug lsn ag* triple mutant is similar to that of *ag* single mutant and *ag lsn* double mutants. The triple mutant flower morphology and organ organization pattern resembled that of *ag*. These genetic data suggested that the *lug lsn* phenotypes are mediated by ectopic *AG* activities. In contrast, the phyllotaxy abnormality caused by *lsn* is not suppressed in the triple mutant (Fig 3-4F).

The *lug lsn ag* triple mutant flowers also showed several unique phenotypes. The triple mutant floral organs are slightly serrated on the edges and the width of the second whorl petals (439 ± 19.6 μm, n = 8) is narrower than that of *ag* single mutant (1106 ± 159.0 μm, n= 8). This phenotype is not simply due to the effects of *lug* because the width of *lsn ag* second whorl petal is 796 ± 57.1 μm (n = 8) and *lug ag* measures 620 ± 29.3 μm (n = 8). Comparison of the organ sizes in these genotypes indicates that *LSN* and *LUG* may interact to determine organ sizes.

Morphology of *lsn seu, lsn ap1*, and *lsn ap2* flowers

Similar to *lug lsn* double mutants, *seu lsn* double mutant plants develop tcf phenotypes (Fig 3-5A-D). The first whorl carpels are not completely fused and often curly, suggesting enhancement of *seu* by *lsn* (Fig 3-5D).

lsn also enhanced carpelloid transformation of first whorl organs in *ap1*. In the early reproductive phase before tcf was developed, sepal number reduction was observed in *ap1-1 lsn-1* (Fig 3-5E, F). Whorl 4 carpel-fusion was always incomplete (Fig 3-5F, H). Sepals of ap1-1 flowers, especially in the auxiliary flowers, are often slightly carpelloid (Fig 3-5G, I, J). This phenotype was enhanced in *ap1-1 lsn-1* flowers (Fig 3-5H). In these flowers, sepals were partially or completely transformed into carpels. Whorl 2 organs were never formed. Whorl 3 organs were occasionally produced. During late reproductive phase, tcf phenotypes caused by *lsn* often appeared with fused whorl 1 carpels (Fig 3-5 K).

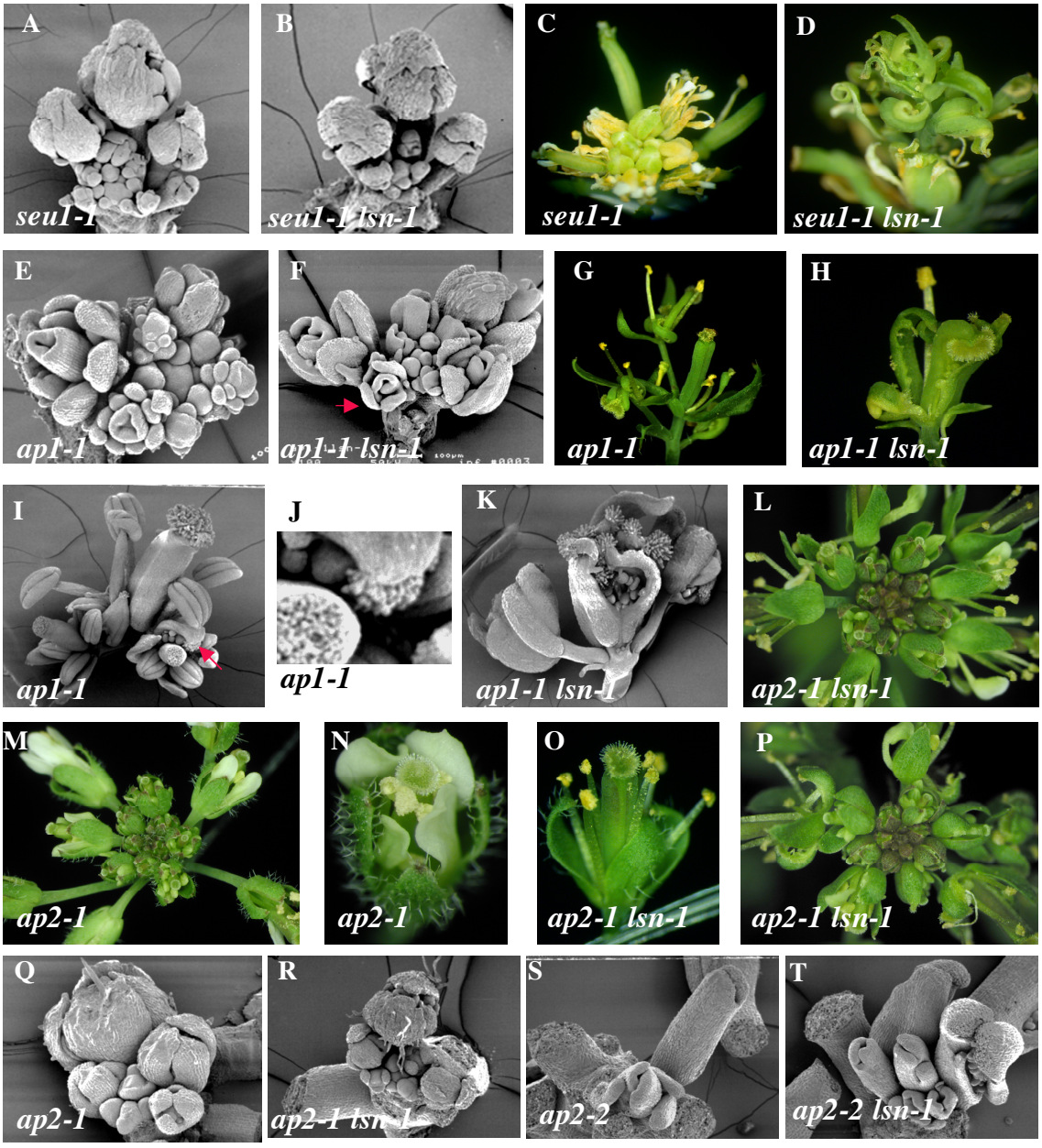


Fig 3-5. Morphological characteristics of *seu lsn, ap1 lsn*, and *ap2 lsn* double mutants at 20 ^{o}C. (A) A *seu-1* inflorescence at the early reproductive phase. (B) A *seu-1 lsn-1* inflorescence at the early reproductive phase. (C) A *seu-1* inflorescence at the late reproductive phase. (D) A *seu-1 lsn-1* inflorescence exhibiting tcf at the late reproductive phase. (E) An *ap1-1* inflorescence in the early reproductive phase. (F) An

ap1-1 lsn-1 inflorescence during early reproductive phase. Red arrow indicates an early stage flower with three sepals. (G) A mature *ap1-1* flower in the early reproductive phase. (H) A mature *ap1-1 lsn-1* flower in the early reproductive phase (I) A mature *ap1-1* flower in the early reproductive phase. Red arrow indicates a slightly carpelloid sepal. (J) Enlarged from (I) showing a slightly carpelloid sepal. (K) A tcf phenotype of an *ap1-1 lsn-1* flower. (L) and (P) different inflorescences showing reduced petal numbers in *ap2-1 lsn-1*. Note that more of the slightly carpelloid sepals were exhibited in P than in L. (M) An *ap2-1* inflorescence. (N) An *ap2-1* flower. (O) A *ap2-1 lsn-1* flower. (Q) An *ap2-1* inflorescence. (R) An *ap2-1 lsn-1* inflorescence. (S) An *ap2-2* inflorescence. (T) An *ap2-2 lsn-1* inflorescence.

Interactions between *ap2* and *lsn* were additive (Fig 3-5L-T). *AG* is strongly ectopically expressed in *ap2-2*. Addition of *lsn-1* alleles into *ap2-2* did not reveal additional morphological defects: *ap2-2 lsn-1* flowers closely resemble that of *ap2-2* or tcf of *lsn-1* (Fig 3-5S, T). Unlike *ap2-2*, *AG* is not ectopically expressed in *ap2-1*. The sepals of *ap2-1* grow branched trichomes at the abaxial surface indicating that these sepals are partially transformed into leaves (Fig 3-5M, N). In *ap2-1 lsn-1* double mutants, petal numbers were reduced to 0-2 in contrast to 3 - 4 in *ap2-1* (Fig 3-5L, O, P). SEM examination did not identify any extra phenotypic differences between *ap2-1* and *ap2-1 lsn-1* (Fig 3-5 Q, R).

Quantitative enhancement of *lsn* by *AG* regulators

In summary of the morphological comparisons, *lsn-1* enhances carpelloid transformation of sepals in *ap1-1* but not in *ap2-1, ap2-2* and *seu-1* at the early reproductive phase. In the late reproductive phase, the tcf morphologies in *lug lsn, ap1-1 lsn-1,* and *seu-1 lsn-1* were reminiscent of *lsn* tcf phenotypes rather than terminating *lug*, *ap1* and seu phenotypes, respectively. To determine whether *lsn* tcf phenotypes are enhanced by these *AG* regulators, %tcf plts and %cf/sht were examined in *ag-1 lsn-1, ap1-1 lsn-1, ap2-1 lsn-1, lug-1 lsn-1*, and *seu-1 lsn-l* plants (Fig 3-6).

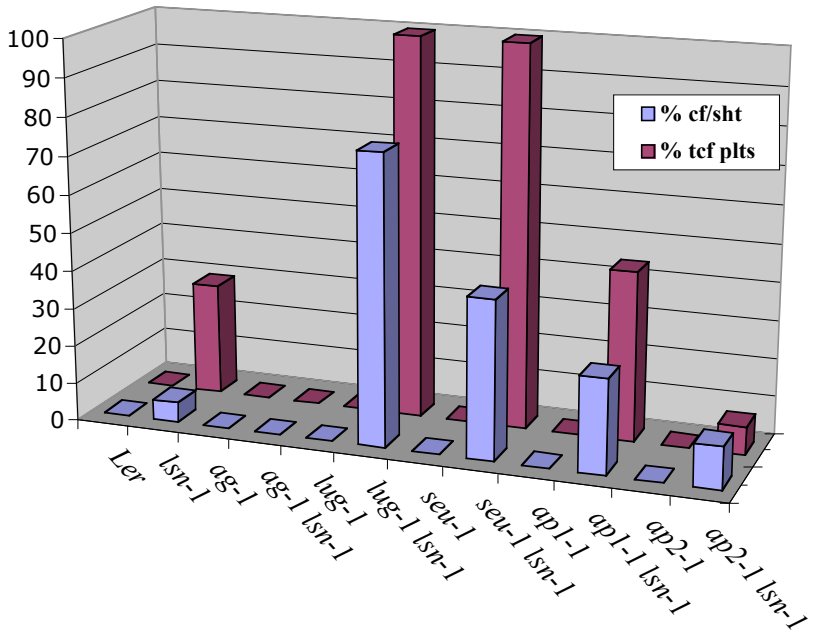


Fig 3-6. Quantification of tcf phenotypes in different mutants at 20 °C. The percentage of plants bearing tcf (%tcf plts) were show in red bars. Amoung those tcf plants, the averaged percentage of carpelloid flowers per shoots (%cf/sht) were presented in red bars. (5- 22 plants were sampled for different genotypes).

Although the small sample sizes did not support testing for statistic significance, the correlating trends between %tcf plts and %cf/sht suggested reliability of the data.

Based on these data, *ap1-1, lug-1*, and *seu-1* enhanced *lsn-1* tcf because *ap1-1 lsn-1, lug-1 lsn-1*, and *seu-1 lsn-1* exhibited more plants that bore tcf and a higher percentage of carpelloid flowers per shoot than the respective single mutants containg *LSN*.

Molecular interactions between LSN and *AG* repressor proteins

Enhancement of *lsn-1* by *seu-1*, *lug-1*, and *ap1-1* encouraged investigations of physical interactions among the respective wild-type proteins. To this end, full-length or truncated cDNAs were cloned into yeast 2-hybrid vectors, fusing to either GAL4 DNA-binding domain or GAL4 activation domain (Fig 3-7A). Transformation of these constructs into an appropriate yeast host, however, did not activate the reporter genes when LSN was tested against AP1, LUG, and SEU (Fig 3-7B). Instead, combinations between AP1 and SEU, and SEU and LUG, LSN and STM, and LSN and KNAT1 activated the reporter genes. Therefore, AP1, LUG, and SEU may form a complex. But LSN did not directly interact with any of these proteins.

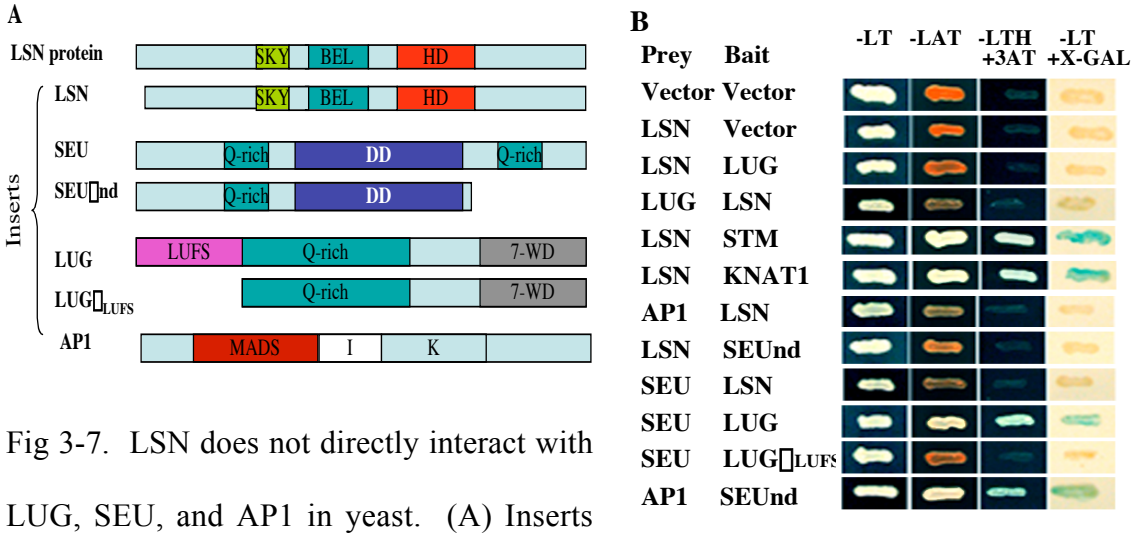


Fig 3-7. LSN does not directly interact with LUG, SEU, and AP1 in yeast. (A) Inserts

used for yeast 2-hybrid constructs. (Protein sizes are not to scale) (B) Reporter gene activities in PJ69-4A cells. –LT, medium lacking leu and trp; –LAT, medium lacking leu ade trp; -LTH + 3AT, medium lacking leu trp his but supplemented with 3 mM 3AT; -LT+X-GAL, medium lack leu trp and followed with lacZ filter assay. Activation of reporter genes showed growth of white colonies on –LAT (adenine reporter) and -LTH+3AT (histidine reporter) medium and blue in –LT+x-GAL assays (LacZ reporter). The interaction between LUG and SEU was established by Mr. Anandkumar Surendrarao (MS thesis, University of Maryland, 2003). The AP1 construct was generously provided by Dr. Beth Krizek (Department of Biological Sciences, University of South Carolina). The STM and KNAT1 constructs were generously provided by Dr. George Haughn (Department of Botany, University of British Columbia, Canada) and were published by Bellaoui *et al*. (2001).

To confirm the results from yeast 2-hybrid assays, an *in vitro* pull-down assays was performed. In this assay, bacterially expressed MBP or MBP-LUG proteins were immobilized onto amylose resin. *In vitro* transcribed/translated LSN proteins, labeled with radioactive ^{35}S-Met, was incubated with the immobilized proteins. BSA/MBP buffer, however, was able to wash away the LSN protein (Fig 3-8), leaving none retained on the column. Therefore, *in vitro* translated LSN did not interact with LUG protein.

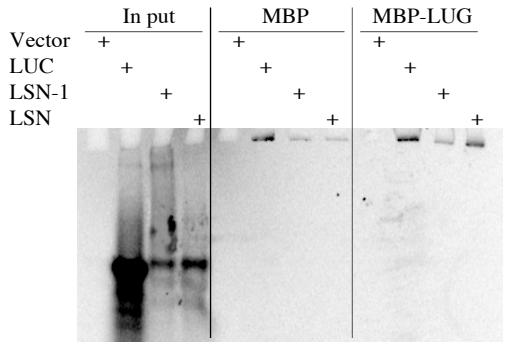


Fig 3-8. LSN does not interact with LUG *in vitro*. LUC, Luciferase; LSN-1, mutsnt ldn protein translated from *lsn-1* mRNA; MBP, moltos binding protein.

DISCUSSION

LSN may have a functionally redundant homolog in *Arabidopsis*

Interestingly, not all *lsn* plants exhibited abnormal floral phenotypes. Although some *lsn* plants that exhibited abnormal flowers, they generated normal flowers in the early reproductive phase. These sporadic and late on-set of floral phenotypes in *lsn* suggested two opposite possible roles for LSN in flower development. One possibility is that LSN is not normally involved in flower development. Mutating *LSN* may have created a neomorphic protein that was poisonous to the normal flower developmental processes. However, this possibility was excluded by observing that the *lsn* null allele also exhibited the same phenotype as the two missense alleles. Therefore, LSN is an important component of *AG* expression regulatory machinery during flower development based on the floral and shoot apical phenotypes exhibited by *lsn* mutants.

Given that LSN is important in normal *AG* regulation, the sporadic tcf phenotype may be due to redundancy in LSN action. *Arabidopsis* probably has a functional substitute for LSN protein (LSN-like), presumably provided by one of the 12 LSN homologs (Fig 3-13), and that the expressivity of *lsn* depends on the ability of LSN-like to substitute for LSN. This ability may become weaker when plants grow older, supported by the late on-set of lsn floral phenotypes in *lsn-3* plants. This ability was also hinderred by *lsn-1* and *lsn-2* alleles that makes mutant proteins. The mutant LSN proteins may have stronger affinities to some of the LSN-associating factors than LSN-like protein and thus, LSN-1 and LSN-2 may compete for the same target DNA binding sites, or co-regulators of *AG,* or both with LSN-like protein. The result is a titration of functional partners or an occupation of target DNA by LSN-1 or LSN-2 proteins and led to stronger phenotypes in *lsn-1* and *lsn-2* plants than in *lsn-3* plants (Fig 3-2). Therefore, the two missense *lsn* alleles are likely recessive antimorphic.

Typically for antimorphic alleles, *lsn-1* and *lsn-2* both show dosage-dependent effects. Two copies of *lsn-1* alleles in homozygous plants produced stronger tcf phenotype than one copy of *lsn-1* allele in *lsn-1/lsn-2* and *lsn-1/lsn-3* plants (Fig 3-2). Similarly, *lsn-2/lsn-2* plants display stronger tcf phenotypes that *lsn-2/lsn-3*. Therefore at least one of the LSN-associating factors, proteins or *lsn*-binding DNA, acts in a dosage dependent manner. The more of this factor that is titrated by LSN-1 or LSN-2, the more severe phenotypes are produced.

LSN acts upstream of a putative *AP1/LUG/SEU* functional complex

Yeast 2-hybrid assays showed that AP1 interacts with SEU, which in turn interacts with LUG via the LUFS domain. These interactions suggest a putative AP1/SEU/LUG protein complex in plants. In such a complex, SEU may act as an adaptor protein to mediate interactions between two or more DNA-binding proteins, such as AP1, in homo- or hetero- protein multimers. At the same time, SEU may also interact with co-regulators such as LUG that exert repressive functions for the complex. However, further analyses are needed to exclude the alternatives that the interactions between each two of AP1, LUG, and SEU may happen in different cells in plants and may participate in different processes.

LSN did not directly interact with AP1, LUG, and SUE. It is, though, still possible that there is another protein, which is not present in the yeast system, mediates the formation of a LSN/AP1/SEU/LUG complex. It is also possible, as seen in other organisms, that post-translational modifications are necessary in order for these proteins to interact in plants. For instance, full-length homeodomain protein UNC-4 of *C. elegans* does not interact with GroTLE ptotein UNC-37 (LUG homolog) in yeast unless the N-terminal arm of UNC-4 is deleted (Winnier et al. 1999). Thus, the stereochemical specificity of the UNC-4 N-terminal arm may determine the interaction *in vivo*. Genetic interactions among *lsn*, *ap1, seu,* and *lug* do not exclude the possibility of LSN/AP1/SEU/LUG complex formation. Hence, molecular interactions among LSN, AP1, SEU, and LUG require further analyses.

However, some molecular and genetic data support an alternative model in which LSN and AP1/LUG/SEU may act in different complexes. Precocious *AG* activities appear much earlier in *lsn* than in *lug seu* double mutants during meristem cell development and specification. In *lsn-1* mutants, *AG* is precociously activated in the shoot apex and causes productions of apical carpelloid flowers (Fig 3-7). In *lug seu* double mutants, *AG* mRNA is ectopically present in the peripheral zone of the inflorescence meristem, where organ primordia are initiated, but is not present at the shoot apex (Franks et al. 2002). The shoot apical meristem gives rises to flower primordia at the peripheral zone. Thus ectopic *AG* is restricted to the floral meristem in *lug seu* mutants but is present in both shoot apical meristems and in floral meristems in *lsn*. Furthermore, AP1 mRNA is never present in the shoot apical meristem (Sessions et al. 2000). Therefore, repression of *AG* in the shoot apical meristem does not require the activities of *AP1*, *LUG*, and *SEU,* but requires *LSN*. Therefore, at least in the shoot apical meristem, LSN is not physically associated with AP1, LUG, and SEU when it represses *AG* expression. Whether or not LSN is part of the putative AP1/LUG/SEU complex in floral organ cells remains unknown.

Initiation and maintenance of *AG* repression during reproductive phase

When plants enter the reproductive phase, *AG* repression by PcG complex (CLF) is overwhelmed by trxG complex (ATX1) (Alvarez-Venegas et al. 2003). However, because of the activities of *LSN*, *AG* is repressed in the pluripotential cells until floral stage 3. Therefore, LSN is a key component of the *AG*-repression initiation

complex that is specified to reproductive shoots to counter the activating-effects of *ATX1* complex.

It appears, overall, that *lsn* and *lug* mutant phenotypes are not identical, and that the *lug lsn* flowers exhibit characteristics of both *lug* and *lsn*. For instance, the horn-like protrusions at the tip of *lug lsn* flowers were reminiscent of *lug* single mutants but the tcf at late reproductive phase exhibited enhanced *lsn* characteristics. Therefore, *lug* and *lsn* are unlikely to be acting in a linear transcriptional regulatory pathway (Fig 3-9A). That is *LSN* and *LUG* may not regulate each other's transcription, or otherwise, *lsn* and *lug* would have exhibited a more similar phenotype. Instead, *LSN* and *LUG* may regulate *AG* in a multiprotein complex (Fig 3-9B), through a parallel redundant manner (Fig 3-9C), or a sequential manner (Fig 3-9D). As was discussed above, no

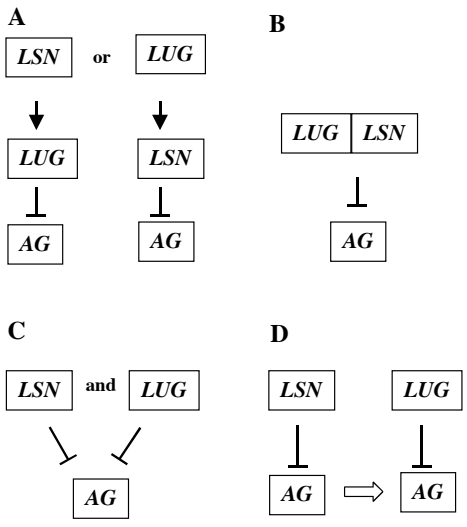


Fig 3-9. Alternative models of *AG* repression by *trans*-acting factors *LSN* and *LUG*. (A) *LSN* and *LUG* regulate each other's transcription in linear regulatory pathways. (B) LSN and LUG act in the same multiprotein complex. (C) *LSN* and *LUG* regulate *AG* in parallel independent pathways. (D) *LSN* and *LUG* regulate *AG* in a sequential manner, in which *LUG* acts later than *LSN*. LUG function is dependent of *LSN* activities.

protein interaction was found between LSN and the pupative LUG/SEU/AP1 complex. Futhermore, LSN acts earlier than the pupative LUG/SEU/AP1 complex. Thus, it is unlikely that LSN and LUG act in the same protein complex, at least in the inflorescence meristem. If LSN and LUG act in complete independent parallel pathways, *lsn lug* double mutant would be expected to exhibit additive phenotypes of *lsn* and *lug*. However, unlike *ap2, lug* enhanced the tcf phenotypes of *lsn* by increasing the number of tcf-bearing plants and number of tcf per plant. Therefore, *LSN* and *LUG* are not acting totally independent of each other. Instead, I prefer the model in which *LSN* and *LUG* regulate *AG* in a sequential manner where the putative LSN complex may modify the chromatin structure in the *AG* enhancer region and may thus establish a "mediating repressive state" upon which the putative LUG complex can exert the functions. This model is analogous to a synthetic pathway in which the "product" of LSN complex is the "substrate" of LUG complex. The function of LUG complex is dependent of the production of the "mediating repressive state" by LSN complex.

The sequential regulatory model is not yet proven, but in a good agreement with the following observations. First, based on the precocious *AG* expression patterns in *lsn* and *lug* mutants, *LSN* acts in the inflorescence meristem, which is earlier than the time *LUG* acts, which occurs in floral organ development. Therefore, the tcf is present in *lsn lug* plants but not in *lug* plants. Second, a putative LSN repression complex is sufficient to repress *AG* in *lug seu* inflorescences. Third, LSN mRNA is absent in the outer two whorls of wild-type flowers, but *AG* is still repressed in the organs arose from these regions. Therefore, a repression maintenance complex is presented in the outer

two whorls, probably in the absence of LSN. Mutating *LUG* results in de-repression of *AG* in the outer two whorls of flowers starting at stage 3. Therefore, *LUG* may be involved in the maintenance of *AG* repression. Third, organs developed from the outer two whorls are abnormal due to ectopic *AG* in *lsn* mutants. Therefore, *LUG* function is not sufficient to repress *AG* when *LSN* is not active in the earlier cell developmental stage in meristems. The similar analyses and conclusions can be reached for the relationships between LSN and AP1 or LSN and SUE.

Together, I propose that LSN establishes *AG* repression in inflorescence meristem and floral meristem and that AP1, LUG, and SEU form a repression complex that maintains *AG* repression in floral organs in the outer two whorls starting at stage 3. A similar example exists in *Drosophila,* where the zinc-finger transcription factor *Tramtrack* (*Ttk*) binds to the enhancer of *engrailed* (*en*) and establishes the repression of *en* in blastoderm stage embryos (Fig 3-10) (Wheeler et al 2002). Following this, Gro and its interactive histone deacetylase Rpd3 maintain the repression when they are recruited to the *en* enhancer by Runt, a transcription factor participates in both initiation and maintenance of *en* repression.

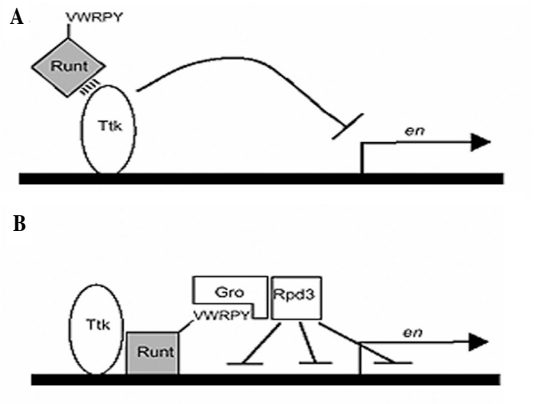


Fig 3-10. Initiation and maintenance of en repression. (A) Ttk recognizes *en* enhancer and establishes repression at the blastoderm embryonic stage. (B) *Trk* interacting transcription factor *Runt* recruits corepressor *Gro*, which in turn brings chromatin-modifying factor *Rpd3* to *en* enhancer and represses *en*. Adapted from Wheeler et al. (2002).

There is a good possibility that AP1 may preferentially recognize *AG cis*-regulatory elements that had been modified by the *LSN* repressive complex in the inflorescence meristem peripheral zone. The interactions between SUE and AP1 may stabilize the AP1/DNA interactions. LUG, the GroTLE family protein, may interact with SEU and bring chromatin-modifying factors to *AG* enhancer to maintain the repression in the floral and floral organ primordia.

The sequential regulatory model is the continuation and completion of the activation barrier model proposed in chapter three. That is, a specific region of plant meristem contains a group of cells that are matured to a specific cell developmental stage. Specific transcriptional repression machinery can act in this region (a barrier) and lead to profound impacts on the decendent cells. The transcriptional regulatory

machineries specific to the decendent cells do not act alone, but along with the inherited impacts from the ancestors to determine the cell type soecificities.

Future tests

The sequential regulatory model can be directly tested *in vivo* (Table 3-3). *AG* mRNA is readily detectable by Northern blot in leaves of *clf-2* (Goodrich et al 1997). *AG* mRNA should be detectable in *clf-2 lsn-1 lug-1* leaves using quantitative RT-PCR. The chromatin structure in *AG* enhancer region can be examined using Chip (Chromatin immunoprecipitation) assay and DNA methylation assays. Introducing a construct expressing inducible *LSN* (*Pi:LSN*) into this triple mutant plant should not change *AG* transcription and chromatin structure in the *AG* enhancer region. Upon induction of transgenic *LSN*, however, *AG* mRNA level may be reduced and the chromatin structure may be altered. Removing the induction should restore *AG* enhancer activities in *Pi:LSN; clf-2 lsn-1 lug-1* transgenic plants. If LUG is only participating in the repression maintenance, a constitutive *LUG* expression construct (Pc_2*:LUG*) in *clf-2 lsn-1 lug-1* leaves will not alter *AG* enhancer activity unless *AP1* and *LSN* are also present in leaves. Whether or not the presence of Pc_1*:AP1* (constitutive *AP1* expression in leaves) affects the maintenance of *AG* transcription may support the role of *AP1* in *AG* repression. However, the direct association of AP1/LUG/SEU repression complex to *AG* needs to be tested using biochemical approaches.

Table 3-3. Examining the initiation and maintenance of *AG* enhancer by *LSN* and *LUG* in *clf-2 lsn-1 lug-1* plants.

transgenes	**NI**	**I**	**RI**
none	A	A	A
Pi:LSN	A	R	A
Pc_1:AP1; Pc_2:LUG	A	A	A
Pi:LSN; Pc_2:LUG	A	R	A
Pi:LSN; Pc_1:AP1; Pc_2:LUG	A	R	R

Expectations are listed as A (activation of *AG* transcription) and R (repression of *AG* transcription). *Pi*, inducible promoter; *Pc*, constitutive promoter; NI, no-induction; I, Induction; RI, removal of induction.

How may growth temperature affect *lsn* tcf phenotypes?

Significant phenotypic differences were observed between *lsn* plants grown at 20 ^{o}C (normal temperature) and 29 ^{o}C (high temperature) (Fig 3-2). Since all three *lsn* alleles including the null exhibited a temperature-enhanced tcf, it is unlikely that a temperature-sensitive mutant protein is responsible for the temperature enhancement. Instead, a proposed LSN functional homolog, LSN-like, may be affected by constant high temperature. Alternatively, *AG* transcription may be regulated by the growth degree-days. These two hypotheses are mutually exclusive.

To grow plants at the high temperature, seeds were germinated at 20^{o}C and then shifted to 29^{o}C when 4 true leaves were produced. Temperature enhancement of tcf seems only occur if plants were shifted up to 29 ^{o}C within two weeks of germination. In addition, when down shifted from 29^{o}C to 20^{o}C, tcf plants continue produce

carpelloid flowers, not reverting to normal flower production. In the other words, once *AG* transcription state is altered from repression to activation, lowering the temperature does not restore its transcription state. Therefore, it seems that the accumulation of heat, not a heat-shock or a change of temperature, determines the *AG* transcription state in *lsn* mutant background. Accumulation of temperature through time (growth degree-day) is proposed to be an important regulatory pathway for plant development based on the thermal clock hypothesis (Poethig 2003). It is possible that heat accumulation regulates *AG* transcription and LSN plays a role in the pathway. Alternatively, temperature is acting at a specific time window of development (during juvenile phase) to assert the effects. To test these hypotheses, *lsn* plants can be exposed under high temperatures at different developmental stages for different periods of time. A positive correlation between the resulted tcf severities and the time period exposed will support the thermal clock hypothesis.

Chapter four

CONCLUDING REMARKS

THE TEMPOTAL CONTROL OF SPATIAL PATTERNS

The above-ground plant body parts are produced by the shoot apical meristem during postembryonic development. The patterning of body parts is determined by the position of the primordia initiation from the meristem. The specification of body parts is determined by the meristem identity genes and the organ identity genes. Temporal control plays an important role in regulating the spatial pattern. Due to the upward growth of stem, activation of certain genes at specific time points in the meristem results in formation of specific lateral structures along the stem. For instances, the rosette leaves are formed at the base of the shoot and the flowers are formed at more apical portion of shoots because the shoot apical meristem changes its identity from vegetative to reproductive phase in *Arabidopsis*. Flowers are separated by internodes because every two sequential flowers are initiated at different times in a meristem. Therefore, temporal control of gene expression is crucial for distinctive spatial patterning in plants.

The temporal control of spatial patterning is evidenced by how *LSN* regulates *AG* at the cellular level. *LSN* expression is peaked in groups of cells in the peripheral zones of inflorescence and floral meristems when the organ primordia are about to form. *LSN* expression is significantly reduced in the sepal primordia. However, a lack of *LSN* activity in the floral meristem peripheral zone in *lsn* mutants causes homeotic

transformation of sepals into carpels due to ectopic *AG* expression. Therefore, *LSN* activity in the peripheral zone may have already imprinted the ancestral cells that later form sepals. The imprint is inherited during somatic cell divisions and passed onto the floral organ specific descendant cells that do not express LSN. This observation demonstrated, for the first time, that floral organs may be "prepatterned" before organ primodia initiation.

At the molecular level, I propose that the temporal regulation relies on the epigenomic activities. The initiation and maintenance of *AG* repression are separated and sequential events. Several known flower-specific *AG* repressors such as LUG, SEU, and AP1 are candidate components of a repression complex that maintains repression of *AG*. In contrast, LSN is responsible for the initiation of *AG* repression in the meristem peripheral zone. This hypothesis is supported by the following observations. First, AP1 physically interacts with SEU, which in turn interacts with LUG, in yeast (Sridhary, Anal and Liu, unpublished). AP1 may recruit corepressors LUG and SEU to *AG cis*-element and may thus provide whorl1 and 2-specificity to the repression complex. Second, while the precociously *AG* expression is observed in the inflorescence metistem in *lsn* mutants, precocious AG expession is only limited in the flower primordia of *lug seu* double mutants. Therefore, *LSN* acts earlier than *LUG* and *SEU* in repressing *AG*. Third, the wild-type *AP1*, *LUG*, *SEU*, and their associated factors are insufficient to determine sepal identity in the first whorl organs of *lsn* mutant flowers. Therefore, the putative AP1/LUG/SEU complex is dependent upon the activity of *LSN*, which acts in the ancestral cells of sepals. Nevertheless, further direct tests are

necessary to determine whether *LSN* is indeed responsible for the initiation of *AG* repression and that the putative AP1/LUG/SEU complex is responsible for maintaining the repression.

LSN EXPRESSION PATTERN DETERMINES PHYLLOTAXY

Recently, two independent laboratories published their studies on *LSN* under different names *PENNYWISE (PNY)* and *BELLRINGER (BLR)* (Smith and Hake, 2003 Byrne *et al*. 2003). Smith and Hake (2003) showed that the inflorescence of *pny* was shorter in stature than wild-type and that the internode patterning of the inflorescence also was affected in *pny* plants. Similar to my study, they also found that *PNY* mRNA was localized in groups of cells that encircle initiating floral primordia in the SAM and later localized to cells in the floral meristem but not in developing floral organ primordial. Genetic interaction was observed between *pny* and *bp* (also known as *KNAT1*). Using *in vitro* pull-down assays, they found that PNY directly interacts with KNAT1 and STM. Based on these data, the authors proposed that the PNY-BP protein complex may function in the inflorescence meristem to form a boundary between lateral organs and the inflorescence meristem and to establish the proper cell orgnization and patterning of the internode. Whereas in flowers, it was proposed that PNY might interact with STM "to control maintenance events in the floral meristem".

Studies of *BLR* showed that a *GUS* reporter gene, driven by a DNA fragment 3447 bp upstream of *BLR* translational initiation codon, was activated in root tip, 8-day-old seedlings, inflorescence meristem, stem, flower pedicel, and in developing flowers

(2003 Byrne *et al*. 2003). Although not discussed by the authors, uneven (patched) GUS staining patterns can be seen in inflorescence and floral meristems. During genetic analysis, *BP* is required in maintaining the SAM in *as1 stm* double mutants*, bp as1 stm* mutants lack a SAM and thus fail to form a vegetative shoot. Similar to *bp as1 stm, blr as1 stm* triple mutants have severely reduced SAM (Byrne *et al* 2003). The paper did not propose a model to explain how *BLR* regulates the phyllotaxy, but it did speculate that the phenotypic defects in *blr*, including aberrant organ initiation patterns, may be in part due to disruption in auxin signaling. It was also pointed out that BLR might have a functional redundancy with another BEL class protein because *blr* has a much milder phenotype than either *bp* or *stm*, the presumed partners of BLR. Protein associations of BLR/BP and BLR/STM were detected by yeast 2-hybrid assays. Since BP is still functional in *blr* mutant, BLR may be substituted by another BEL protein (probably At2g27990).

Combining the data from these two studies and the data described in this thesis, two functions may be proposed for *LSN* in the inflorescence meristem. First, LSN may interact with STM in the cells located at the boundary between the central zone and peripheral zone of inflorescence meristem, where the expression of *STM* and *LSN* overlap. One possible function of this interaction may be to define the maturation stage of meristem cells and thus the size of the central zone, in paralelle to the *CLV-WUS* feedback loop. Second, interaction between LSN and KNAT1 may define the position and boundary of flower primordia in cells that express both *LSN* and *KNAT1*. A loss-of-function mutation in *LSN* may cause defect in the developmental program of cells

and positioning of primordia. The key to test these hypotheses is to define each of the transcriptional regulatory pathways by isolating more genetic components, especially the direct targets of the LSN/STM or LSN/KNAT1.

ARE MADS BOX GENES THE TARGETS OF *LSN*?

A fourth study by Roeder *et al*. (2003) describes the role of *LSN*, under the name of *REPLUMLESS* (*RPL*), in replum development. *rpl* was isolated as an enhancer of *fruitful* (*ful)*. *rpl ful* double mutant lacks the replum. Close inspection showed that the replum cells were replaced by narrow files of cells that resemble cells found in the valve margin in *rpl*. *RPL::RPL-GUS* reporter gene analyses (construct information is not accessible) and *in situ* hybridization with a *RPL*-specific probe showed that *RPL* is expressed in the replum of developing ovaries. GT140 and *SHP2::GUS,* two molecular markers known to be expressed in stripes at the valve margin, are ectopically expressed in the replum region of the *rpl* mutants. Therefore, RPL likely acts as a transcription factor in the replum to negatively regulate the expression of MADS box genes *SHATTERPROOF1,2* (*SHP*), thereby preventing the replum from adopting a valve margin cell fate.

The study of *LSN* by Roeder *et al* (2003) and the study described in this dessertation suggest a possible role of *LSN* in repressing some MADS box genes such as *AG* and *SHP*. However, the LSN-binding sites identified by EMSA and sequese alignment among *AG* orthologs in Brassicaceae were not found in the cis-regulatory region of *SHP*. Whether *LSN* regulates *SHP* indirectly or directly by binding to *SHP*

cis-elements that differ from LSN-binding sites in *AG cis*-regulatory region is not known. This question is worth pursuing in the future to evaluate DNA binding specificity and affinity of *LSN*. It is possible that the DNA-binding specificity and affinity of *LSN*, and thus its biological functions, are dependent upon the other transcription factors that physically interact with LSN.

Chapter five

MATERIAL AND METHODS

MATERIAL

lsn-1

The *larson-1* (*lsn-1*) allele was isolated by Dr. Joshua Levin in an ethyl methanesulphonate (EMS) mutagenesis screen for modifiers of *ufo* in L*er* (Levin *et al.* 1998). It was suspected to be a weak allele of *lug* for its occasional narrow petals. However, it was found that *lsn-1* complemented *lug*. Dr. Levin generously offered Dr. Liu the *lsn-1* mutant seeds for further analysis.

lsn-2

To obtain more alleles of *lsn*, TILLING (Targeting Induced Local Lesions IN Genomes) was conducted to identify mutations in *LSN* in Col ecotype (McCallum et al 2000a, McCallum et al 2000b). Specifically, a pair of primers (5'CAAGCTGCCAT CTTCATGCTGATT and 5'CGCTTGCAACACGACATCTCCA AG) was designed to amplify a region of *LSN* genomic DNA predicted by CODDDEL software to have a relatively high possibility of being mutated to nonsense and missense mutations by EMS. These primers were used in Dr. Steven Henikoff's laboratory to PCR amplify the *LSN* DNA from pooled genomic templates extracted from EMS mutagenized M2 plants. The products were separated by the Li-cor gel system that detects mismatches in heteroduplexes created by melting and annealing of heteroallelic DNA. Specific DNA

samples were identified as candidates for containing mutations in *LSN*. I sequenced a total of 12 candidate PCR products to verify the presence of mutations. Two lines of seeds (ABRC stocks CS86187 and CS87355) were obtained from Dr. Henikoff because the mutations were located in an exon (exon II). Phenotypes of these plants were examined under both 20°C and 29°C for two generations. CS86187 did not exhibite any visual phenotype. Mutants that produced visual phenotypes (CS 87335) were kept and designated as *lsn-2* for further analysis. Allele specific CAPS marker (mlsn-2) (Table 5-1) was used to examine cosegregation of *lsn-2* allele and phenotypes in the F1 and F2 outcrosses.

lsn-3

A collection of Ds transposon induced mutant lines in L*er* ecotype background was generated in Dr. Robert Martienssen's laboratory (Sundaresan *et al*. 1995). The sequences flanking the Ds elements are available as a searchable database to public at http://genetrap.cshl.org. *LSN* cDNA sequence was used to screen for Ds insertion using the BLAST search. A candidate line (ET6411) was obtained and designated *lsn-3*. The Ds insertion can be detected by PCR using three primers (mlsn-3), one located in the Ds element and two in the *LSN* gene (Table 5-1). PCR products were sequenced to determine the exact insertion site of the Ds element.

METHODS

Growth conditions

Seeds were planted on the surface of sterilized soil or MS-agar plates supplied with water and treated at 4^{o}C for 4 days. Plants were then geminated and grown at 20 ^{o}C or 29 ^{o}C with continuous light and 50 - 60% humidity.

Genetic crosses

For genetic crosses, stamens were detached from the donor flowers and used to pollinate the stigma of recipient flowers, which had been emasculated at floral stages 8 to 10 (staged based on Smyth *et al*. 1990). Genotypes of F1 or F2 plants were identified with phenotypes (such as in *ag-1 lug-1*), allele-specific PCR markers (such as in *lsn-1 lsn-3, lsn-2 lsn-3, lsn-2 lsn-3*), or both (such as in *KB9*; *lsn-1, ag-1 lsn-1, lug lsn, lsn-1 seu-1, ap1-1 lsn-1, ap2-1 lsn-1*). To eliminate possible contamination from self-fertilization, allele-specific molecular makers (Table 5-1) were used to identify the maternally inherited alleles when paternal phenotypes were obvious.

RT-PCR to assay *lsn-3* mRNA

Total RNA was extracted from *lsn-3* flowers using Tri-reagent following manufacturer's instructions (Sigma). Reverse transcription was performed using oligo dT_{20} (0.5 □g/□l) primer in the presence of SuperScript II reverse-transcriptase (Invetrogen). PCR used the reverse transcribed first strand cDNA as the template and *LSN* cDNA specific primers (TCATCATGCTCCTGTTTGGA and TGTTGCTGTTGTCAGGGAAG) and ACTIN2 cDNA specific primers

(GTTGGGATGAACCAGAAGGA and CTTACAATTTCCCG CTCTGC) to examine presence of mRNAs.

Molecular markers

The PCR-based molecular markers located at the close approximate to *lsn-1* on chromosome V or within *lsn* are listed in Table 5-1.

Table 5-1. Molecular markers located near the *lsn-1* locus.

Markers	**Left primers**	**Right primers**	**PCR (bp)**	**Enzymes**
F7J8 (CAPS)	TGCTGTTAAACCCAACCAAA	GAGTTGTGGCTCGACGAAAT	750	Hae III/ ler
T10O8 (CAPS)	CCCGAATCCTTCTTTTTCATT	TGCTTTGAAAAGACCGGATAA	690	RSA I/Ler
T20L15 (CAPS)	AAACGAAACCAGGGACTGTG	GGGGTACCTGGATCCTGAAT	691	Fok I/col
T7H20-14K (CAPS)	CACAGACCCACTCACTGCAT	GGGCTTTGTTCTTCCTCCTC	331	Mnl I/ler
T7H20-30K (SSLP)	CGTTGGCTTTTGAAGATCAC	TCCCATGAGAGGGAAAGGTT	155	Col
T7H20-53k (SSLP)	ATTGACCCAAAAGTTCCTCTAG	TGCACAATGATTTACAAAGTGAAC	123	Col
T7H20-60K (CAPS)	CCGGAGATTAGCCTCTTCCT	CGACCGGTGATTCCAAAG	506	Hinf I/ler
T22P11 (CAPS)	CCAGCTATCAACCTGGGGTA	TCTCCGATTCGAATTGTGCT	803	Cla I/col
F15A17 (CAPS)	TCGGTTCAGGTGTCAATCAA	TTGCATCACCTTCTCAATTCC	699	Hha I/col
mlsn-1	GCCACAACAACAAGATCCAACA	AGAAATGATCGAAGAGCCAAGC	185	Taq I/lsn-1
mlsn-2	GGAAGTGATAGTTCTAGAGGCT	CAGTCGACATGTCCCATCTAA	382	Dde I/wt
mlsn-3	ACCCGGGACTTTTCACTTTT ACCCGACCGGATCGTATCGGT	CACCGAGAAATCCCATGAAA	1000 440	wt *lsn-3*

Names of markers represent the BAC clones containing the primer sequences. mlsn-1, 2, and 3 are allele specific markers for *lsn-1, 2,* and *3*. Endonuclease that cleave the specific alleles are listed when apply.

Map-based cloning of *lsn-1*

lsn-1lug-2 L*er* (Landsburg *erecta*) was crossed to wild-type Col (Columbia) plants to generate the mapping population. Leaf DNA samples from 95 *lsn-1 lug-2* double mutant plants in the F2 mapping population were analyzed with PCR-based CAPS or SSLP markers (Konieczny & Ausubel 1993, Bell & Ecker 1994). The PCR primers, the restriction enzymes, and the expected products are listed in Table 5-1. Sizes of PCR/restriction digestion products were analyzed with agarose gel electrophoresis, stained with ethidium bromide and observed and documented with IS-500 Gel Documentation System (Alpha Innotech Corperation).

Recombinants between closely linked markers within centimorgans to *lsn-1* were selected for finer mapping. When *lsn-1* was mapped to a region of BAC (bacterial artificial chromosome) clone T7H20, candidate genes that had been annotated by AGI were sequenced to identify the *lsn-1* mutation. A CAPS marker (mlsn-1) (Table 5-1) was designed for detecting the putative *lsn-1* to examine cosegregation between putative *lsn-1* mutation and the mutant phenotypes.

Leaf DNA extraction for PCR-based analyses

Using sharp forceps, 2-3 small (or 1 large) leaves from individual plants were collected, and immersed in 400 ul of Edward extraction buffer (200 mM Tris pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5% SDS) in 1.5 ml eppendoff tubes. The tissues were ground with a pestle and allewed to sit at room temperature until all samples were ready. The tubes were then centrifuged at 14,000 rpm for 2 minutes at room-

temperature. The supernatant (350 µl) was transferred to a fresh tube containing 350 µl of isopropanol. Samples were then mixed and centrifuged for 5 minutes at 14,000 rpm. The pellet containing DNA was washed with 350 µl of 70% ethanol and air dried for 10 minutes. The DNA was then dissolved in 100 µl water and stored at 4 ^{o}C for PCR analyses.

Microscopic analyses

Plant parts were dissected under Zeiss Stemi SV6 dissecting microscope and photographed using a Nikon COOLPIX 995 digital camera. Scanning electro microscopy (SEM) samples were collected, fixed, and coated based on the procedure of Bowman *et al.* (1989). Samples were examined on an AMRAY 1000A scanning electron microscope. Images were captured on a Polaroid camera.

cDNA library screen for full-length *LSN* mRNA

A recA^{-} E. coli host strain XL1-Blue was grown in LB broth supplemented with 0.2% maltose. Late log-phase cultures were harvested by centrifugation at 4,000 g for 10 minutes. After one wash with a 0.2% maltose solution, cells were resuspended in 10 mM $MgSO_4$ at OD_{600} of 1.0. A Ler flower cDNA library packed in Uni-ZAP XR λ phage was provided by Dr. Detlef Weigel. 5.5 x 10^4 phage particles were inoculated in 100 µl of bacteria at 37 ^{o}C and incubated for 15 minutes. The cultures were then mixed with 7.5 ml of 0.7% molten agar supplemented with 0.2% maltose and spread on LB plates (150 mm). The plate was air dried for 20 minutes and then incubated at 37 ^{o}C overnight. A total of 10 such plates were inoculated.

The next day, the plates were chilled at 4 ^{o}C for 1 hour. Two dry nitrocellulose membranes were used to sequentially lift the phage plaques on each plate. Specifically, the membrane was placed on the plates for 30 – 90 seconds and then peeled off. Membranes were immersed in a DNA denaturation solution (1.5M NaCl, 0.5N NaOH) for 5 minutes, then in neutralization buffer (1M Tris, pH 7.4, 1.5M NaCl) for 5 minutes, and finally in 2x SSC for 5 minutes. Membranes were UV cross-linked and probed with a ^{32}P radiolabeled *LSN*-specific probe. The 602-bp DNA fragment of *LSN* was PCR amplified using primers 5'-TCCATCTCTCGATTCCCACT and 5'-CATCAGA AACACCGCA GAGA and then labeled with random labeling kit (Roche) . Two positives on the duplicated membranes were selected for phagemid excision. To achieve this, the positive phage plaques were picked from the plate and resuspended in 500 μl SM buffer (0.1M NaCl, 0.008M $MgSO_4$, 1M Tris.Cl, pH 7.5, 0.01% gelatin) and a drop of chloroform. The suspension was incubated at room temperature for 2 hours. 200 μl of this suspension was added to 200 μl of XL1-Blue cell (OD_{600} = 1.0) in 10 mM $MgSO_4$ along with 1 μl of R408 helper phage (1 x 10 ^{6} pfu/ml). After incubation for 15 minutes at 37 ^{o}C, 3 ml of LB was added and the incubation continued for 3 additional hours. 30 μl of this culture was spread on a LB agar plate containing 50 μg/ml of ampicillin and incubated overnight to obtain *LSN* cDNA clonies in pBluescript SK plasmids (Fig 5-1A). The rest of the suspension was heated at 70 ^{o}C for 20 minutes and centrifuged at 4,000 g for 5 minutes. The supernanant containing the phagemid was stored at 4 ^{o}C.

Mapping the 5' end of *LSN* mRNA using RACE

Total RNA was extracted from wild-type Ler flowers using Tri-reagent (Sigma) following the manufacturer's protocol. 5'-RACE was conducted using5' RACE System for Rapid Amplification of cDNA Ends (Gibco, Version 2.0). Briefly, first strand cDNA was reverse-transcribed using a *LSN* gene specific primer (CATCAGAAACAC CGCAGAGA) located in the first predicted exon in the presence of SuperScript II reverse-transcriptase (Invitrogen). Three nested *LSN* gene specific primers (ATCGACGCGTAGCCCGTGAA, AACAACACCGGGAACG ACTA, and AAGTCC GGCGTTAAAGGAAT) were used to pair with the forward universal primer in three nested PCR reactions. The longest products from the third round of PCR were cloned into pCR II-TOPO using TOPO TA cloning kit (Invitrogen) and the sequenced. Only one type of sequence was obtained, and it is 6nt longer at the 5' than the full-length cDNA clone (pFLL).

Construction of *lsn* mutant cDNAs

Total RNA was extracted from *lsn-1* flowers using Tri-reagent (Sigma). Reverse transcription was performed using the primer 5'CCTAACGTCAAAGACACT CCTCCGTTTGT in the presence of SuperScript II reverse-transcriptase. A PCR, using the first strand cDNA as the template and primers 5'TCCATCTCTCGATTCCCACT and 5'TGTTGCTGTTGTCAGGGAAG, produced a 1kb-product and was cloned into pCR II-TOPO vector (Invitrogen). Two plasmids from separate colonies were sequenced and contained no mutation other than *lsn-1*. The above plasmid DNA was digested with Bgl II and used to replace the Bgl II fragment in the pFLF plasmid, which

contains a full-length wild-type LSN cDNA (Fig 5-1B). Resulting plasmids were examined for insert orientation and the presence of *lsn-1* mutations using a *lsn-1* specific CAPS marker (PCR with primers 5' GCCACAACAACAAGATCCAACA and 5'CCTAACGTCAAA GACACT CCTCCGTTTGT followed by Taq I digestion). This procedure yielded a full-length *lsn-1* cDNA clone (pFMF) and a 1 kb-deletion mutant clone (pDMF).

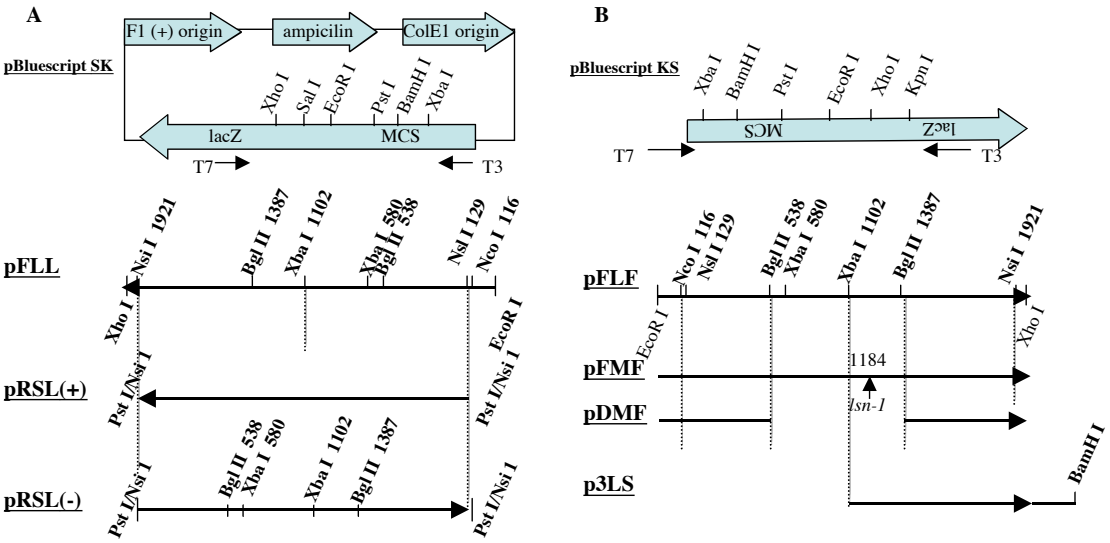


Fig 5-1. Basic subcloning constructs. (A) pBluescript SK based constructs. (B) pBluescript KS based constructs. Constructs names are listed on the left. Nco I site is located 1 bp upstream of *LSN* translation initiation codon. 3' Nsi I site is 78 bp downstream of *LSN* translation stop codon. pFLL and pFLF, full-length *LSN* cDNA; pRSL(±), subclones of full-length *LSN* cDNA for various translational fusions; p3LS, transcribes sense *LSN* probe using T7 promoter; pFMF and pDMF, mutant *lsn* cDNA clones. See appedix I for more details on these constructs.

In vitro transcription/translation of *LSN*

The wild-type cDNA (pFLF) and *lsn-1* cDNA (pFMF) were used to produce LSN and LSN-1 proteins *in vitro*. Purified DNA templates were added to the TNT® T7 Coupled Wheat Germ Extract System (Promega) following the manufacturer's protocols. [^{35}S]-Met was added to the reaction to label proteins synthesized. To purify the protein products, the reaction mixture was first passed through self-packed Sephadex G50 (2 ml) (Sigma) columns, which were packed in 3 ml syringes with column buffer (20 mMTris-HCl, pH 7.6, 150 mM NaCl), and pre-ran with 0.5 ml BSA (0.5 mg/ml in the column buffer). Fractions of 0.2 - 0.3 ml were collected for each sample, including controls (reactions using pBluescript KS DNA and *luciferase* cDNA templates). The fractions with highest radioactivities were saved in storage buffer (50% glycerol, 20 Mm Tris-HCl, pH 7.5) at -20 ^{o}C. All fractions from pBluescript KS were saved and the fractions with the same retention time to the LSN samples were used in the pull-down assays.

RNA *in situ* hybridization

The protocol was modified from that of Meyerowitz (1987). Briefly, plant tissues were harvested and fixed for 20 minutes in a fixative mix (50% ethanol, 5% acetic acid, and 3.7% formaldehyde) under vacuum (10 min at 12 in^3 Hg). These were washed one more time with the fixative for another 40 minutes. Through ethanol series, tissues were dehydrated to 100% ethanol with 15% ethanol increment at each step. A final concentration of 0.3% Eosin-Y was added to the 95% and 100% ethanol steps. Ethanol was then replaced with xylenes using 25%, 50%, 75% xylenes mixed with

ethanol and 100% xylenes. After 3 repeats of 30-min 100% xylenes washes, 50% volume of paraplast-plus chips (Fisher) were added to xylenes solvent, which melted the paraplast chips at 42^{o}C for 30 min. Molten paraplast (100%) was used to infiltrate the tissues 7 times at intervals of 4 hours to overnight at 56 ^{o}C. These infiltrated samples along with the paraplast were poured into Petri plates to solidify at room temperature for overnight. The tissues were sectioned at 8 micrometers. Ribbons were arranged onto Superfrost microscope slides (Fisher) and baked at 42 ^{o}C overnight.

Microscope slides prepared as described above were soaked in 100% xylenes twice (10 min each) to dissolve the paraplast. The sections were re-hydrated through an ethanol series (100% to 30% ethanol with 5% to 15% decrease in each step) containing 0.85% NaCl for 2 minutes at each step. Then these sections were treated with proteinase K (1 □g/ml) for 30 minutes followed by post-fixation by 4% of paraformaldehyde (pH 7.0) for 10 minutes. After dehydration through an ethanol series again, samples were air-dried and were ready to hybridize with RNA probes.

A ~800 bp fragment at the 3' end of *LSN* cDNA was obtained by digesting pRSL (+) plasmid with Xba I (Fig 5-1A). This linearized pRSL(+) transcribes an anitisense RNA probe using a T7 promoter. An antisense RNA probe was transcribed from the BamH1 linearized p3LS plasmid using the T7 promoter (Fig 5-1A). Transcriptions were performed using the DIG RNA Labeling Kit (Roche) following the manufacturer's protocols.

Hybridization was carried out in a hybridization solution containing 10 μl of 10x in situ salts (3 M NaCl, 100 mM tris pH 6.5, 100 mM Na-phosphate buffer, pH 6.8, 50 mM EDTA), 40 μl deionized formamide, 20 μl of 50% dextran sulfate, 2 μl of 50x denhardts solution, 1 μl of tRNA (100 mg/ml), 7 μl of H_2O, and 40 μl of mRNA probe (3 ng/ul in 50% formamide) for each slide at 56 ^oC for 24 - 36 hours. Samples were washed in 0.2x SSC at 56 ^oC for 15 min each and blocked with 1% Boehringer block for 30 min at room temperature. AP-conjugated anti-DIG antibody, diluted to 1:1250 in BTNT (1% BSA, 100 mM Tris pH 7.5, 150 mM NaCl, and 0.3% Triton x-100), was incubated with the samples for 2 hour for the immunological reaction to take place. After washing away the free antibodies with BTNT, the sample pH was brought up to 9.5 with alkaline buffer (Tris pH 9.5, 100mM NaCl, and 50 mM $MgCl_2$). Substrates (Western Blue from Promega) were provided to the conjugated AP in an excessive amount (~12 ml) in a mailer box containing 5 slides for 1-3 days until lue/purple color appeared on the positive controls.

Production of recombinant proteins in bacteria

LSN cDNA excised from pRSL (+) using BamH I and EcoR I restriction enzymes was cloned in to pGEX-3x bacterial expression vector (Amersham Pharmacia Biotech) to create a pGEL construct, in which GST is translationally fused to the N terminus of LSN at the BamH I site. (Fig 5-1). The juction between GST and LSN was confirmed by sequencing. pGEL encodes the recombinant GST-LSN protein, with three amino acid residues (MAD) deleted from the N-terminus of LSN. pGEL and pGEX-3x plasmids were transformed into BL21 host E. coli cells (Clontech). The

transformants were grown at 37 ^{o}C overnight in a small volume (5 ml) of LB broth containing ampicillin (100 ng/ml) and chloramphenicol (15 ng/ml). This culture was used to inoculate 1L of ampicillin/chloramphenicol/LB broth the second day. After 3-hour growth at 37 ^{o}C in a shaker at 250 rpm, isopropylthio-β-D-galactoside (IPTG, 0.1 mM) was added and the culture was transferred to 29 ^{o}C with vigorous shaking (300 rpm) for 5 hours. Cells were harvested at 4500g and washed once with ice-cold Tris-HCl (20 mM, pH 7.5). Protein was extracted using BugBuster^{TM} protein extraction reagent (Novagen). The insoluble part of the protein extract was washed 3 times with 1x IB wash buffer from the protein refolding kit (Novagen) and stored in Tris-HCl (20 mM, pH 7.5) containing 60 % glycerol at –80 ^{o}C.

Two days before the LSN protein was needed, every 10 mg (wet weight) of stored protein was washed 3 times with IB wash buffer and then dissolved with 1 ml of solubilization buffer (1x IB Solubilization buffer, 0.3% N-lauroylsarcosine, 1 mM of DTT) at room temperature (Novagen). This mixture was clarified by centrifugation at 12,000 g for 10 minutes at 4 ^{o}C. Solubilized proteins were dialyzed with Tris-HCl (20 mM, pH 8.5) in the presence of 0.1 mM DTT twice at 4 ^{o}C. The third and fourth dialysis was done at pH 7.5 without dithiothreitol (DTT). A proteinase inhibitor cocktail (1x, BD Biosciences Pharmingen) was used at each step after cell lysis. Protein quantity and quality was estimated with SDS-PAGE gel, western blot, and Bradford assays. Negative controls were established using the same procedures but either omitted the addition of IPTG during 29 ^{o}C incubation, or replaced pGEL with pGEX-3x plasmids. Refolded protein samples were stored at –20^{o}C in storage buffer (50 mM

NaCl, 20 mM Tris-HCl, pH 7.5, 0.1 EDTA, 200 μg/ml BSA, and 50% glycerol) and used within one week after refolding.

Agrobacterium mediated plant gene transformation

Plant transformation was based on a protocol from Dr. Marty Yanofsky's laboratory. Briefly, the primary shoots of newly bolted plants were clipped off to encourage the growth of secondary shoots. At the fourth day after clipping, the inflorescences of these plants were immersed in the infiltration media (0.5x MS saltes, 1x B5 vitamens, 5% sucrose, 0.044 μM benzylamino purine, and 0.03% Silwet L-77) containing Agrobacterium harvested at late log phase. Vacuum was applied for 10 min at 12 Hg/in^3 to facilitate infiltration of the agrobacterium. Pots containing the infiltrated plants were laid on the side and were covered with Saran Wrap overnight. On the next day, plants were placed upright and grown to produce seeds. T1 seeds were collected and sterilized before geminating on selective MS-agar plates. Specifically, seeds were first washed once in 0.05% tween-20 and subsequently in 70% ethanol. Seeds were subsequently rinsed with sterile water for three times and then planted on MS-agar plates containing a selective agent, such as kanamycin, against non-transformed plants. These seeds were vernalized for three days at 4 ^oC and then geminated and grown at 20 ^oC for 15 days. Plants with 2-4 fully developed leaves were candidate transformants and were transferred to soil for further growth and analysis.

Electrophoretic mobility shift assays (EMSA)

Radioactive DNA fragments were produced by modified PCR reactions (15 cycles of extension) in which cold dATP was used at 160 μM final concentration (2 μl of 1.6 mM stock per 20 μl reaction) along with 3.3 μl of [^{32}P]dATP (Amersham Pharmacia Biotech, cat # PB10384) . Pairs of primers were used to amplify the *AG* DNA from the second intronic region (Table 5-2). The PCR products were separated using a 6% TBE-PAGE gel. After identifying the expected band with autoradiography, the band was excised from the gel and immersed in 400 μl of TE buffer overnight at room temperature. DNA molecules were diffused into the TE buffer during this time.

Table 5-2. Primer pairs used for amplifying DNA from the second intron of *AG*.

Forward primers	Reverse primers	Product size (bp)
CGTAGAAATGGTTTGCTCAAGA	CAACAATGGAGGATGGATGA	870
TTGTGATCATCCATCCTCCA	ATCTTGCGCTCAATTCCAAC	917
GTTGGAATTGAGCGCAAGAT	CAACAACCCATTAACACATTGG	904
TGGTCTGCCTTCTACGATCC	TTAATTTCTGCCACCGATCC	872
TTGTGATCATCCATCCTCCA	GCTTTTCTTGTTTTGGACATGA	747
CTAATTCGACACGCAATTTCCA	ATCTTGCGCTCAATTCCAAC	363
CCACCAAAACATAAAACATGTCAA	ATCTTGCGCTCAATTCCAAC	300
TCATGTCCAAAACAAGAAAAGCT	ATCTTGCGCTCAATTCCAAC	193
CCGATGGGTACTCTACGAAAT	ATCTTGCGCTCAATTCCAAC	100
TCATGTCCAAAACAAGAAAAGCT	ATTTCGTAGAGTACCCATCGG	93

In a 10 μl reaction, 1μl of radioactive DNA from above, 2 μl of 5x binding buffer (5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT , 250 mM NaCl, 50 mM tris-HCl pH 7.5, 0.25 μg/μl poly dIdC, and 20% glycerol), 1 μl of BSA (100 ng/ul), and 2 μl of

protein extract (~2-4 ng/μl of GST-LSN and 20-40 ng/μl of unknown bacterial protein) were mixed and incubated on ice for 30 minutes. If the DNA probe is longer than 500bp, 0.0002 units of DNaseI were added to each binding reaction and incubated for additional 15 minutes at room temperature (DNaseI protection assay). Samples were loaded onto a 6% DNA retardation gel (Invetrogen) and ran 80 minutes at 180 volts in 0.5x TBE running buffer. Gels were placed on a piece of Whatman 3 MM filter paper and wrapped with a plastic wrap. The gels were then exposed to phosphorimager screens overnight. Data were collected using a *Storm*^{TM} Gel and Blot Imaging System (Amersham Biosciences) installed with an ImageQuant TL software.

GUS staining

Plant tissues were harvested into ice-cold 90% acetone and incubated for 20 minutes. After washing with 10 ml freshly made staining buffer (0.2% triton X-100, 50 mM $NaHPO_4$ buffer, pH 7.2, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide) for 20 minutes on ice, the samples were placed in fresh staining buffer containing 2 mM of X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt). Vacuum (10 min at 12 Hg/in^3) was applied to the samples, which were then incubated in the dark at 37^{o}C for 16 hours.

Stained samples were dehydrated by ethanol series with increments of 15% starting at 20% and ending at 100%. The 50% ethanol step was substituted with FAA (50% ethanol, 5% formaldehyde, and 10% acetic acid). 0.3% of Eosin-Y was added to the 95% and 100% ethanol steps to counter-stain the tissues. Ethanol was replaced

from the tissue by tert-butanol through a series of tert-butanol/ethanol mixture containing 25%, 50%, 75%, and 100% of tert-butanol. Following two 100% tert-butanol washes, 50% (v/v) paraplast was added to the tert-butanol and incubated overnight at 56 ^{o}C. Afterwards, 100% of the melted paraplast was used to infiltrate the tissues 4-5 times each with 4-hour incubations at 56 ^{o}C. Finally, the embedded samples were poured into Petri dishes to solidify overnight at room temperature. Embedded samples were sectioned with microtome to 6 micrometer sections. Ribbons arranged onto supperfrost/plus microscop slides (Fisher), de-waxes with two xylenes washes at 10 min each, and mounted for light microscopy and photography.

Yeast 2-hybrid assays

Full-length or truncated *LSN* cDNA were cloned into yeast 2-hybrid bait vectors pGBT9 and pGBKT7 or prey vectors pGAD424, pGADT7 (Clontech). The vector/insert junctions were sequenced to confirm the correct fusion. These constructs were transformed as pairs of bait and prey into yeast host PJ69-4A using protocols previously described (Gietz and Woods 2002, James *et al.* 1996). Briefly, yeast cells were inoculated into 250 ml of liquid 2x YPAD medium (Clontech) and incubated on a rotary shaker at 200 rpm at 30°C. When the OD_{600} reached 1.0 (~1 x 10^6 cells/ml) in about 24 hours, cells were harvested by centrifugation at 6,000g for 5 min. These cells were inoculated into 50 –100 ml of per-warmed YPAD medium and allowed to grow in a 200 rpm shaker at 30°C for about 4 hours. Cells were harvested when the titer reached 2 x 10^7 cells/ml (~4 hours) and were resuspended in about 1- 2 ml of cold lithium acetate solution (10 mM) so that the final cell titer is 1 x 10^9. This cell

suspension was dispensed to pre-chilled microtubes (10^8/tube) and stored on ice (for less than 15 minutes). These tubes were centrifuged at 14,000g for 30 seconds to harvest cells right before the addition of transformation mix. 240 μl of PEG 3500 was overlaid on the top of the cells in each tube. Then 120 μl of transformation mix containing a pair of bait and prey plasmids (300 ng each), boiling-denatured salmon sperm DNA (100 μg), and 100 mM of LiAc was added to the top of the PEG layer. When all transformations were prepared, contents of each tube were mixed by pipetting. Tubes were left at room temperature when all tubes were prepared at this step (~ 30 minutes). The tubes were then heat-shocked at 42 ^{o}C for 45 min. Cells were harvested from each tube by a quick spin at 14,000g for 30 seconds. Sterile water (0.5 ml) was added to each tube after removing the supernatants. Cells were resuspended gently in water and spread on auxotrophic selective plates (-leu –trp) for vector proliferation at 30 ^{o}C. Two to three days later, the colonies were transferred to selective plates (-leu –trp –ade or –leu –trp – his) to examine reporter gene activities.

In vitro pull-down assays

Bacteria produced MBP and MBP-LUG recombinant proteins (construct was provided by Dr. Joanne Conner) was immobilized onto amylose beads and washed with column buffer (20 mM tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT). This protein was refolded by Dr. V.V. Sridha and had been shown to be active in a different assay. [^{35}S] Met labeled LSN protein extracts were incubated with the preloaded amylose beads (3 μg protein per 100 μl beads) at 4 ^{o}C for three hours. Beads were washed two times with 6 column volumes of column buffer supplemented with 0.5

mg/ml of BSA. Beads were eluted twice with 200 μl each with column buffer supplemented with maltose (10 mM). The eluates (400 ul per reaction) were precipitated with a 10% final concentration of TCA (trichloroacetic acid) on ice for 30 minutes. The precipitates were centrifuged at 12,000g for 5 min and then washed with 200 μl of cold acetone. After air-dried, pellets were resuspended in 15 μl of SDS-PAGE loading buffer (Invitrogen) and heated for 15 min at 65 ^{o}C before loading. The gel (4-16% gradient, Invitrogen) was exposed to a phosphor image screen for 3 days after electrophoresis.

AG sequence alignment

The *AG* enhancer DNA sequences from 29 Brassicaceae species and 12 non-Brassicaceae species that were published by Hong *et al*. (2003). These sequences were analyzed using a perl script written by Mr. Yongpan Yan, a Ph.D candidate at Center for Advanced Research in Biotechnology, University of Maryland Biotechnology Institute (Rockville MD 20850) (unpublished). This program used a 24-mer sliding-window with steps of 1-base and maximum of 6-base mismatches per window to search for conserved sequence motifs within the ~3 kb *AG* enhancer regions at both orientations.

Appendices

Appendix I: Constructs used in this dissertation.

Table A1-1. The constructs used in this dissertation.

Construct names	Donor of insert	Excise sites	Recipient vectors	Applications
pBSL*	BAC T7H20	Spe I / BamH I (Predigest with Bstx I) (the 8.99 kb band)	pBlueScript SK	Subcloning for rescue
pBIL*	pBSL	Spe I / BamH I	pBIN20	Genomic rescue
pFLL#	cDNA library	EcoR I / Xho I	pBlueScript KS	cloning
pFLF#	pFLL	EcoR I / Xho I	pBlueScript SK	Subcloning
pRSL(±)#	pFLL	Nsi I	pBlueScript SK (Pst I)	Translational fussion
pSFL	pFLL	EcoR I / Xho I	pSMB	Overexpression in plant
pGAL	pRSL(+)	BamH I / Sal I	pGAD424	Yeast expression (AD-fussion)
pGEL	pRSL(+)	BamH I / EcoR I	pGEX-3x	GST fusion
pGBL	pRSL(+)	BamH I / Sal I	pGBT9	Yeast expression (BD-fussion)
pBFL	pFLL	Nco I / Xho I	pGBKT7 (Nco I/ Sal I)	Yeast expression (BD-fussion)
pALF	pRSL(+)	BamH I / Sal I	pGADT7 (BamH I/Xho I)	Yeast expression (AD-fussion)
p3LS#	pRSL(-)	Xba I / BamH I	pBlueScript KS	Sense LSN probe using T7
pADN	PRSL (+)	81 aa deletion at the N-terminal of LSN	pGAD424 (BamH 1/Sal 1)	Yeast expression (AD-fusion)
pADT14	pADN	See Fig A1-1c	pGAD424	Yeast expression (AD-fusion)
pADT4	pADN		pGAD424	Yeast expression (AD-fusion)
pADT6	pADN		pGAD424	Yeast expression (AD-fusion)
PADT13	pADN		pGAD424	Yeast expression (AD-fusion)
pTA-prod5	5’ RACE		PCR II-TOPO	5’ sequwncing
pFML#	pFLF		pBlueScript SK	*LSN-1* cDNA
pDML#	pFLF	Bgl II	pBlueScript SK	LSN HD deletion

The recipients used the same restriction endonuclease as the donors unless otherwise indicated. # Restriction maps were shown in Fig A1-1. Restriction maps were shown in Fig A1-2. * Restriction maps were shown in Fig A1-3

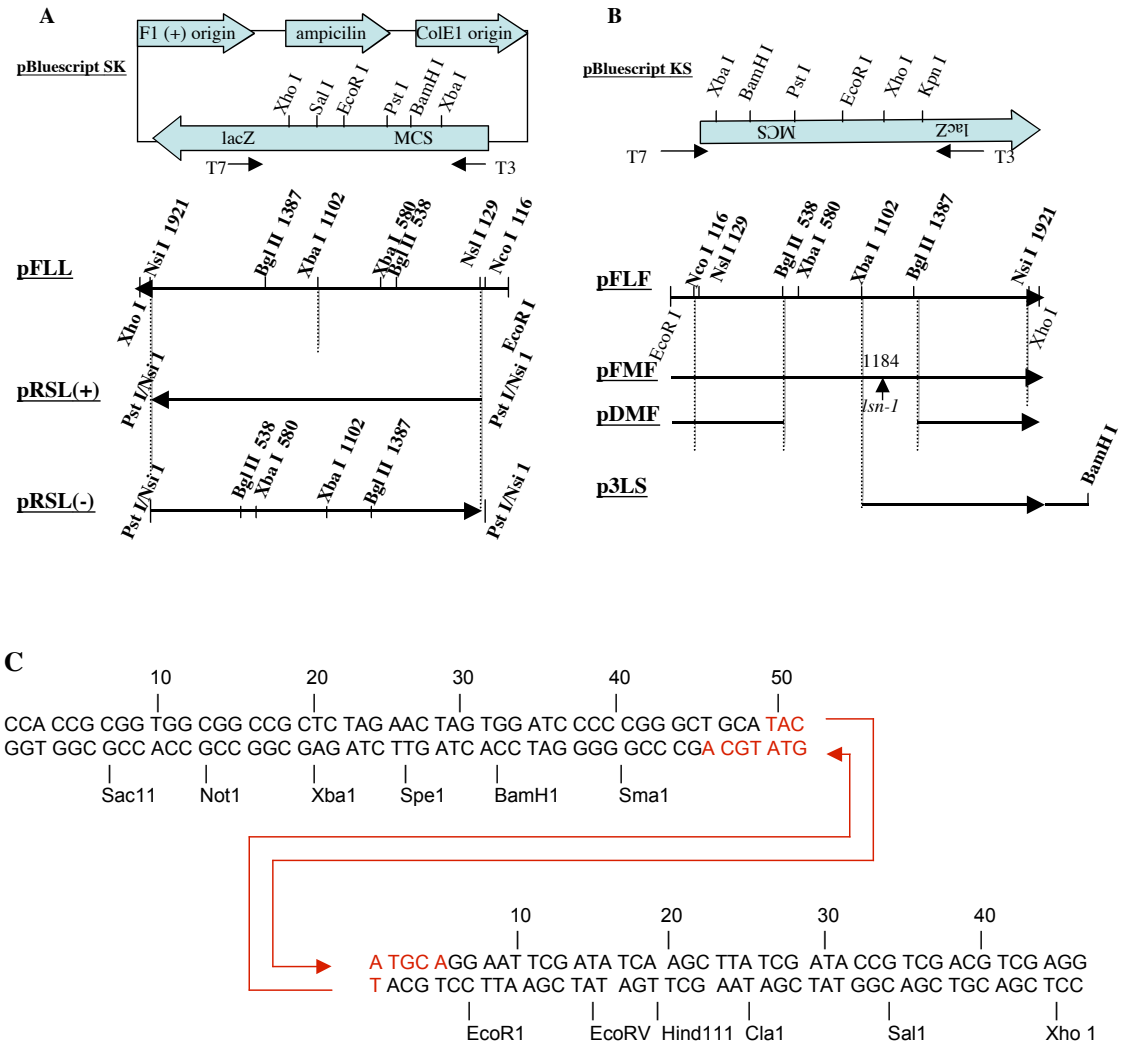


Fig A1-1. Clones and sub-clones of *LSN* cDNA. The insertional sites of each construct was indicated at the ends of the horizontal maps. The arrows on the horizontal maps indicate the sense orintation of *LSN* cDNAs. All constructs contain the endogenous stop codon of *LSN*. A. Orintations of LSN cDNA clones in pBluscript SK. Note that pFLL and pFLF contain the same LSN cDNA, but transcribe into antisense and sense mRNA, respectively, using T7 promotor. pRSL(+) and pRSL(-) contain the same cDNA, but the transcribe antisense and sense mRNA of LSN, respectively, using T7

promotor. The 5' end of LSN cDNA was deleted at Nsi 1 site in pRLS(±), results in deletion of MAD at the N-terminal of LSN protein when translated. Pst1/Nsi 1 indicates the chimaric site resulted from ligation between Pst 1 digested vector and Nsi 1 digested insert. B. Orintations of LSN cDNA clones in pBluescript KS. The p3LS sequence between the arrow and BamH I was the sequence between Pst I and BamH I in pBluescript SK vector. C. The sequence of the multicloning sites in pRSL (±). The red lines indicated *LSN* insert. The red letters were the ends of *LSN* produced by digesting pFLL with Nsi I. The triplets were in-frame with LSN.

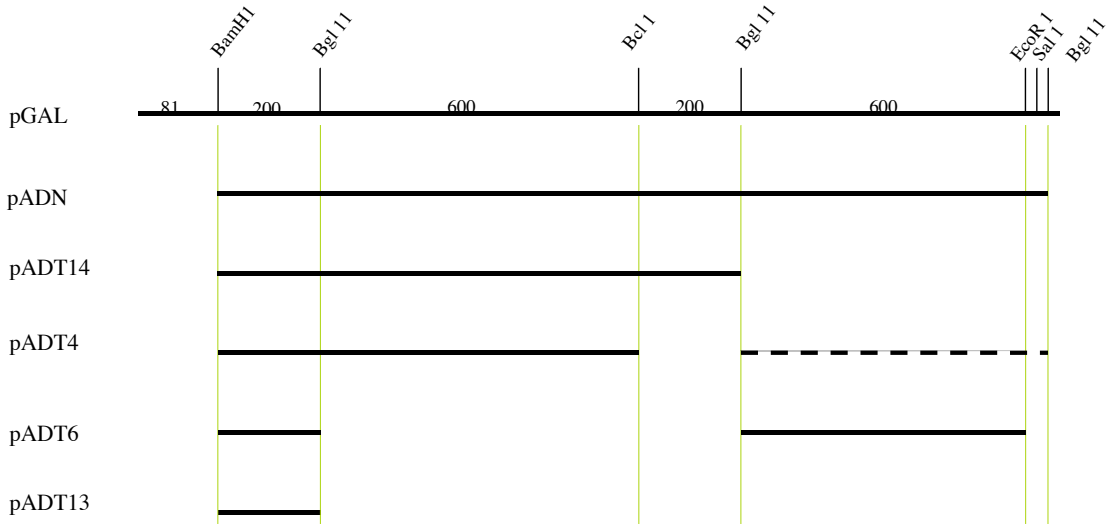


Fig A1-2. Additional yeast two-hybrid constructs. A LSN cDNA was PCR amplified from pFLL plasmid DNA template using T7 primer and N-D78 primer (CGGGATCC CTTCTTCTTCTTCATCCACCGCA) with an engineered BamH 1 site. The PCR product was cuted with Sal I and BamH I and then fused to the GAL4 activation domain in pGAD424, deleting N-terminal 81 aa residues from LSN. Partial diggestion with Bgl II and Bcl I followed by self-ligation resulted in the pADT constructs that contain different truncations of LSN. The solid lines indicate inframe fusions. The dashed line indicated a frameshift.

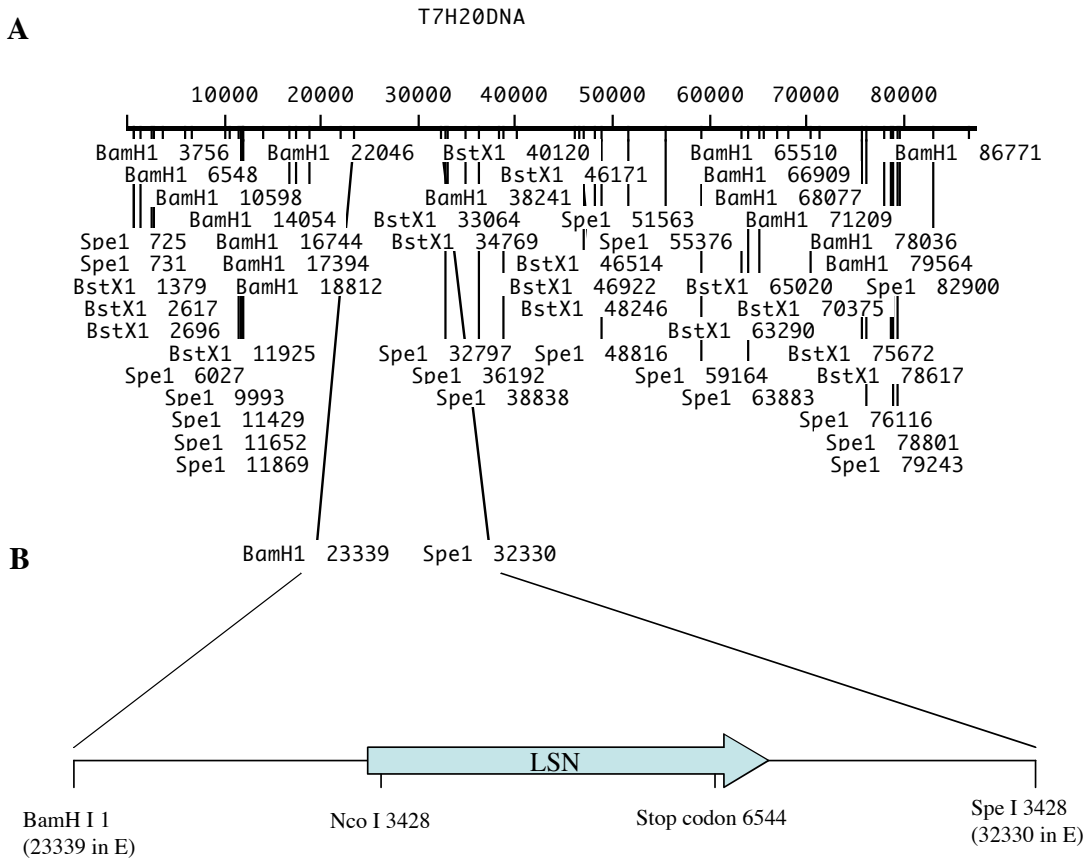


Fig A1-3. The restriction maps of T7H20. (A) T7H20 digested with BstX I, BamH I, and Spe I. The fragment contained the *LSN* open reading frame was ~9kb (red bar) and was the longest in all fragments. (B) The insert of pBSL and pBIL constructs. This fragment was excised from T7H20 with a pair of BamH I and Spe I restriction sites. The red arrow indicated the LSN open reading frame. Nco I indicated the *LSN* translational initiation site. This open reading frame ended at 6544 bp in relevant to the BamH I site.

Appendix II: Rabbit □-LSN antiserum development.

Two peptides located at the N-terminal (CYEPYHVLQQSRRDKLR) (lot # 31445) and C-terminal (NRQFGRDFIGGSNHQC) (lot# 31446) ends of LSN were synthesized in conjugation with KLH using Cysteine by Genemed Synthesis, Inc (http://www.genemedsyn.com) (South San Francisco, CA). These peptides were used because they were predicted to exhibit relatively high hydrophilicity, which usually indicates high antigenecity, than the other regions of the protein. It was also predicted by Genemed Synthesis, Inc that residuals in these peptides were likely exposed, allowing easy access of the antibodies. The antigens were used to immunize individual rabbits in a ten-week protocol (two injections). Two rabbits were immunized for each antigen.

At the end, 5 ml/per rabbit of pre-immune sera and 100 ml/rabbit of antiserum were obtained. These antisera did not recognize LSN protein produced by plants or bacterium in various Western blots. Fig A2 shows one of the typical western-blot using these antisera.

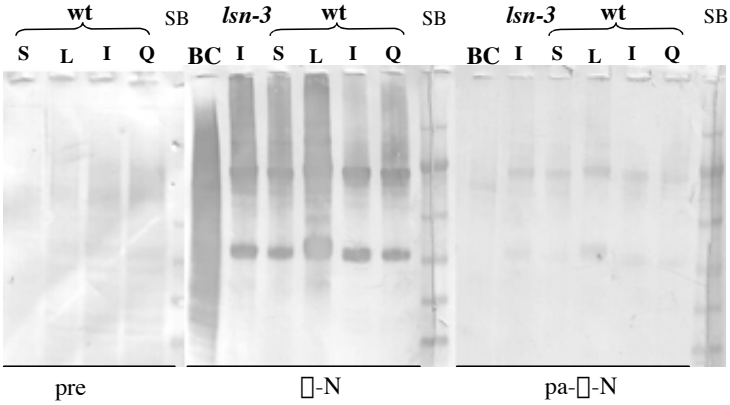


Fig A2-1. □-LSN antisera recongnized non-specific plant proteins. Pre-immune sera (pre), □-LSN antisera (□-N), and □-LSN

antisera that were pre-absorbed by the acetone powder of *lsn-3* inflorescences (pa-□-N) were used at 1/1000 dilution to examine protein extractions from bacteria (BC) and plant tissues. □-LSN antisera did not recognize any specific protein. I, inflorescence; S, stem; L,leaf; Q, silique; SB, See-Blue protein standards from Invitrogen.

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