Molecular Genetics of Meristem Development in *Arabidopsis thaliana*

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Abstract of Dissertation

Molecular Genetics of Meristem Development in *Arabidopsis thaliana*

Post-embryonic growth in flowering plants is accomplished by meristems, groups of undifferentiated cells that persist as stem cells and initiate organs. The shoot apical meristem (SAM) contains slowly dividing cells that are surrounded by more mitotically active cells which produce organs. In *Arabidopsis*, the SAM is initiated during embryonic development, then initiates leaves and flowers. The SAM remains the same size throughout the life span of the plant suggesting that the rate of cell proliferation is balanced with the incorporation of cells into organ primordia. Mutations in the *CLAVATA1 (CLV1)* gene result in plants that have enlarged apical and floral meristems, indicating an imbalance in proliferation and differentiation in these structures.

The *CLV1* gene encodes a protein with a putative extracellular domain containing 21 leucine-rich repeats (LRRs) and a cytoplasmic ser/thr kinase domain. *In vitro* experiments show that the CLV1 kinase domain autophosphorylates, possibly by an intermolecular mechanism, on serine residues. The *CLV1* gene is expressed in the center of the SAM and young floral meristems but not in organ primordia, suggesting that CLV1 may receive a signal that regulates cell proliferation in the central regions of meristems.

KAPP (kinase-associated protein phosphatase), binds and dephosphorylates the CLV1 kinase domain *in vitro*. The *KAPP* gene is expressed throughout SAM and young floral meristems, including all *CLV1* expressing cells. Transgenic plants overexpressing *KAPP* have a phenotype similar to weak *clv1* alleles, and transgenic lines that reduce *KAPP* function partially suppress the *clv1-4* mutant phenotype. The KAPP protein likely functions in several pathways but appears to be a negative regulator of CLV1 signaling.

Many other LRR-kinases have been identified from *Arabidopsis* . A gene of unknown function, called *CLavata1-Like4* (*CLL4*), is specifically expressed on the flanks of the SAM, in a pattern complementary to *CLV1*. The *ERECTA* gene, which was defined by mutations that affect plants height and floral organ shape, is expressed throughout the SAM and in expanding floral organs. Therefore, members of the LRR-kinase gene family may play a variety of roles during plant developmental.

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INTRODUCTION

Genetic Control of Apical and Floral Meristem Development in *Arabidopsis*

Robert W. Williams

Plants do a balancing act. They have evolved structures and developmental processes that allow a great degree of plasticity and response to the environment all within a non-motile organism encased in a rigid cellulose cell wall. With different habits and separate evolutionary lineages, several aspects of plant development are not shared with animals. During plant embryonic development, only a simple body plan is laid down; most patterning and organogenesis occurs post-embryonically. Furthermore, there are no cell or tissue migrations during plant morphogenesis. Plants therefore rely on regulated cell division, cell expansion and cell death to produce an organ or tissue (Steeves and Sussex, 1989).

Continuous plant growth and development is largely the result of the activity of meristems. Meristems are collections of undifferentiated stem cells that renew themselves and initiate specific organs and/or tissues throughout the life span of the plant. During embryogenesis, the root meristem and the shoot apical meristem (SAM) are established which produce the mature root and aerial portions of the plant, respectively. The two meristems, while they do not resemble each other, are highly structured and organs/tissues of specific identity are initiated in very stereotypical patterns. Ablation, transplantation and mosaic studies show that both meristems rely largely on positional cues, rather than lineage relationships, for proper patterning (Steeves and Sussex, 1989; van den Berg et al., 1995, 1997). Therefore, there are likely many intercellular signals that regulate cell proliferation, expansion and identity.

Shoot Meristems

Post-fertilization, the *Arabidopsis thaliana* zygote undergoes many rounds of mitosis and forms a heart shaped embryo with apical-basal polarity. The two regions of the embryo are separated into tiers. The lower tier forms the embryonic stem and basal root meristem (Figure 1A; Scheres et al., 1994). The root meristem is an ordered collection of cellular

initials that produce daughter cells of specific identities (Dolan et al., 1993). The apical region (or upper tier) of the embryo forms the shoot apical meristem (SAM) in between two leaf primordia, called the cotyledons (Figure 1A). The mature embryo desiccates and becomes dormant. Upon seed germination, the SAM becomes active and functions as a pool of undifferentiated cells that divide and produces the leaves, stem, vascular tissue and flowers.

The SAM consists of three clonally distinct cell layers (Figure 1B). The outermost L1 layer contains cells that undergo anticlinal cell divisions, that is the plane of cell division is perpendicular to the surface of the plant, so that L1 cells produce L1 daughter cells. L1 derived cells make up the epidermis of the mature plant. The L2, which lies immediately beneath the L1, also contains cells that undergo anticlinal cell divisions. L2 derived cells produce the subepidermal tissues of the plant. The L3 provides the bulk of the meristem and consists of cells that undergo anticlinal and periclinal (plane of cell division is parallel to the surface of the plant) cell divisions. Therefore, the L3 appears much less organized than the L1 or L2. While the three layers are largely isolated, occasionally a periclinal cell division can produce a daughter that is forced into another cell layer and the resulting cell adopts the fate of its new position (Steeves and Sussex, 1989).

Superimposed on this layered structure of the SAM, is another pattern of zonation (Figure 1C). The central zone (CZ) consists of slowly dividing cells. This is surrounded by the peripheral zone (PZ) where rates of mitosis increase and organ primordia are initiated. As the CZ cells divide, their daughters enter the PZ where they become incorporated into organ primordia. Though not known, one possible significance of the CZ/PZ structure of the SAM is that the CZ cells correspond to undifferentiated stem cells and the PZ corresponds to cells that are differentiating (Steeves and Sussex, 1989). The overall size and shape of the SAM is maintained during the life of the plant indicating that the rate of cell division in the CZ and the rate of cells incorporated into primordia in the PZ are tightly coupled.

The SAM is a site of continuous organogenesis. Vegetative development is characterized by the production of leaf primordia in a spiral pattern with very little elongation between successive leaves (Figure 1D). The leaves begin as bulges on the flanks of the SAM and are likely the result of increased periclinal cell divisions in the L3 (reviewed in Poethig, 1997). In response to age and environmental cues, the SAM undergoes a phase change and begins producing flower primordia (Figure 1E). After the transition to flowering, the SAM maintains its' size, shape and structure but now produces floral primordia, with greatly increased internode elongation, in the same spiral pattern as it did when making leaves. During both the vegetative and reproductive phases, organogenesis is limited to the flanks of the SAM so the meristem as a whole remains indeterminate.

Floral Meristems

The young floral primordia is itself a meristem, similar in structure to the SAM. The floral meristem (FM), which is thought to be a modified SAM, has the same cell layering and zonation as the SAM (Figure 1C; Steeves and Sussex, 1989). Unlike the SAM, though, the FM initiates floral organ primordia in a whorled pattern and the FM is a determinant structure. Floral organs are initiated on the flanks of the FM except for the central floral organs, the carpels, which are initiated on the top of the FM, thus ending meristematic growth (Smyth et al., 1990)

The FM initiates specific numbers of floral organs with defined identity in concentric whorls. The outerwhorl initiates four sepals, that enclose the developing flower bud. Four petals are formed in the second whorl, interior to the sepals. The third whorl consists of six stamen and the internal fourth whorl initiates two carpels that fuse to form the terminal gynoecium (Figure, 1F; Smyth et al., 1990). All floral organs have regular and

reproducible spacing along several axes, indicating that the location, rates and planes of cell division are tightly regulated in the FM.

Genetic Dissection of SAM and FM Initiation and Maintenance

Many genes have been identified in a variety of plant species that alter aspects of SAM and FM development. As mentioned above, the FM is thought to be a modified SAM, thus many genes that control SAM development are likely to have similar functions in the FM. Though *Arabidopsis* will be emphasized here, many of the genes discussed below have identified orthologs/homologs in other plants species (table 1) and in many cases the *Arabidopsis* genes were cloned based on sequence similarity to the previously isolated genes from another species.

The *Arabidopsis SHOOTMERISTEMLESS* (*STM*) gene is necessary for the proper formation of the embryonic SAM and meristem maintenance during post-embryonic growth. *STM* was originally identified by a mutant that did not form a SAM; however, the root, hypocotyl and cotyledons are normal (Barton and Poethig, 1993). Plants homozygous for weak *stm* alleles can form small disorganized SAMs that terminate early. Adventitious shoots can form and even produce flowers, but the floral meristems terminate prematurely with fewer numbers of floral organs (Clark et al., 1996; Endrizzi et al., 1996). This is also brings up an interesting question about the origin of cotyledons in *stm* mutants. Are they formed independently of the SAM, or is the SAM consumed during the production of the cotyledons?

The maize *Knotted1* (*Kn1*) gene was among the first meristem function genes to be cloned. *Kn1* encodes a homeodomain containing protein (Vollbrecht et al., 1991) that is expressed in the corpus of the meristem but is excluded from initiating organs (Jackson et al., 1994). The *Kn1* locus was initially characterized by a series of dominant alleles that cause ectopic cell divisions along the leaf veins (Freeling and Hake, 1985). The dominant

knotted leaf phenotype was later shown to be the result of ectopic expression of the *Kn1* gene in leaf veins (Smith et al., 1992). Loss-of function *Kn1* alleles also have defects in meristem maintenance similar to the phenotype seen in *Arabidopsis* homozygous for weak *stm* alleles (Kerstetter et al., 1997). The *Arabidopsis STM* gene encodes a homeodomain protein very similar in sequence and expression pattern to *Kn1* (Long et al., 1996).

The exact cellular function of *Kn1/STM* is not known. For example, wild-type *STM* could promote meristematic cell divisions and in the absence of *STM* function, organ initiation occurs at the top of the meristem thus incorporating all of the meristematic cells. Or, *STM* could be required to keep cells in an undifferentiated state. No doubt the interplay between cell division and differentiation at the SAM is quite complex. It is also interesting to note that *Kn1* is expressed only in the corpus where periclinal cell divisions occur and ectopic expression of *Kn1* in barley awns induces periclinal cell divisions and the production of ectopic floral meristems (Williams-Carrier et al., 1997). Perhaps one function of the *Kn1* gene is to promote periclinal cell divisions, though *Kn1* RNA is excluded from organ primordia where there is an increase in periclinal cell divisions so there might be other genes that control the planes of cell division during organ formation.

Several other genes in *Arabidopsis* have been identified that superficially have similar mutant phenotypes to *stm*. Mutations in the *WUSCHEL* (*WUS*) gene result in plants that have cotyledons but fail to grow and initiate leaves properly (Laux et al., 1996). Adventitious shoots can form, and occasionally flowers with reduced numbers of floral organs are formed. Unlike *stm*, *wus* mutant plants form an SAM but it does not grow indicating that *WUS* is required more for the growth of the meristematic cells than in the production of the SAM (Laux et al., 1996). As such, *WUS* is likely to control patterns of cell division.

Mutations in the *ZWILLE/PINHEAD* (*ZLL*) gene result in plants that produce a single terminal organ, usually a pin-like structure or a leaf, in place of the SAM. Interestingly, adventitious shoots can form that produce normal looking leaves and flowers

(McConnell and Barton, 1995; Moussian et al., 1998). The *ZLL* gene may only be required during an early stage of SAM development to establish a pool of undifferentiated cells, and this stage can be by-passed during the production of adventitious shoots.

The *ZLL* gene encodes a protein of unknown function, although proteins with sequence similarity are found in the animal kingdom. *ZLL* is expressed in the vascular tissue throughout the life span of the plant, but is also expressed briefly in the embryonic SAM (Moussian et al., 1998). Therefore, the *ZLL* gene product may function either in the vascular tissue to produce a signal/substance that maintains the SAM, or it is required only transiently in the embryonic SAM to set-up a self-perpetuating pool of meristematic cells.

Several other mutants have been characterized that reduce or eliminate SAM function. *Arabidopsis cup-shaped cotyledon* (*cuc*) mutants also lack a SAM and the cotyledons are fused (Aida et al., 1997), which is similar to mutations in the petunia *NO APICAL MERISTEM* (*NAM*) gene (Souer et al., 1996). The Petunia *NAM* and the *Arabidopsis CUC2* genes are members of a plant specific gene family of unknown function (Souer et al., 1996; Aida et al., 1997). Interestingly, *NAM* is not expressed in the SAM proper but in a ring of cells surrounding the SAM and in-between organ primordia (Souer et al., 1996). Therefore, these genes likely act in a cell non-autonomous manner to promote meristem growth and in a cell autonomous manner to establish boundaries between organ primordia.

Many genes have also been identified that are required to maintain the size of the SAM and FM but are not required for meristem initiation. The best characterized are the *CLAVATA* (*CLV*) loci in *Arabidopsis*. Recessive *clv1*, *clv2* or *clv3* alleles result in plants with enlarged apical and floral meristems (Leyser and Furner, 1992; Clark et al., 1993; Clark et al., 1995). This ultimately leads to stem fasciation and the production of supernumery floral organs (Figure 2A-B). The meristems in *clv* mutants still have the characteristic three cell layers (Figure 2C-D; Clark et al., 1993), indicating that many aspects of SAM structure and cell-cell communication are still intact. Thus, the *CLV* genes

normally function to limit the size of the SAM and FM. Like *STM*, the exact function of the *CLV* genes is unknown. They could either repress the rates of cell divisions in the CZ or they could promote organogenesis in the PZ. More than likely, those two processes are closely regulated.

The *CLV1* gene encodes a putative receptor-kinase (Figure 2E) that is expressed in the corpus of the SAM and FMs but is excluded from organ primordia (Figure 2E-F; Clark et al., 1997). The expression pattern is most consistent with *CLV1* regulating rates of cell proliferation in the CZ, but a role in promoting differentiation in the PZ cannot be ruled out. Furthermore, the L1 does not express *CLV1* although it overproliferates to maintain the cell layering suggesting that the superficial layers can sense the size of the underlying L3 and divide accordingly in a *CLV1*-independent manner. The ligand for the CLV1 receptor is unknown, though the *CLV3* gene has recently been cloned and the sequence suggests a small protein with an N-terminal signal sequence, so it is a good candidate (Jennifer Fletcher, Ruediger Simon, Mark Running and Elliot Meyerowitz, unpublished data).

No downstream components in the CLV1 signaling pathway have been identified although the kinase-associated protein phosphatase (KAPP) is a likely negative regulator of the pathway. *KAPP* has a much wider domain of expression but it includes all *CLV1* expressing cells. *In vitro*, the KAPP protein can bind and dephosphorylate the phosphorylated CLV1 kinase domain. Plants overexpressing *KAPP* have a weak *clv* phenotype suggesting an *in vivo* relevance of the *in vitro* data (Williams et al., 1997).

A tomato mutant, called *fasciated* (*f*), has enlarged apical and floral meristems and extra floral organs. The wild-type *f* gene may therefore play a role in tomato meristem development similar to how the *CLV* loci act in *Arabidopsis*. Interestingly, mosaic analysis indicate that a mutant L3 layer is sufficient to express the *f* phenotype (Szymkowiak and Sussex, 1992). Thus, there must be some cell-cell communication such that the L1 and L2 sense the rates of cell division in the L3 and respond accordingly.

The *Arabidopsis fas1*, *fas2*, *mgoun1* and *mgoun2* mutants share a similar enlarged SAM phenotype, often leading to stem fasciation (Leyser and Furner, 1992; Laufs et al., 1998). Unlike the *clv* mutants, all four of these mutants have defects in organ initiation. Specifically, there is a reduction in the numbers of leaves and floral organs that are produced (Leyser and Furner, 1992; Laufs et al., 1998). So these genes are good candidates for being involved in organogenesis on the flanks of the SAM.

The analysis of double mutant *Arabidopsis* plants has begun to put some of the genes discussed above in a larger framework. *clv1 clv3* double mutants are no more severe than strong *clv1* or *clv3* single mutants. Furthermore, reduction of *CLV3* dosage can enhance the semi-dominance of the *clv1-1* allele (Clark et al., 1995). Taken together, it is possible that the *CLV1* and *CLV3* gene products function in the same pathway, which is consistent with the recent molecular cloning of *CLV3*. It is not yet known if *CLV2* may function in the same pathway as *CLV1* and *CLV3*.

clv1 stm and *clv3 stm* double mutants have characteristics of both single mutant phenotypes. In particular, the double mutants do not properly form an embryonic SAM, but enlarged adventitious shoots are formed at a high frequency that bare flowers with increased numbers of floral organs. These fasciated shoots, however, can still terminate early (Clark et al., 1996). This suggests that the *CLV* genes still function in a *stm* background and vice versa. So the *CLV* genes and *STM* likely function in separate pathways, but still may be regulating the same processes. Conversely, *wus* is epistatic to *clv1* (Laux et al., 1996) suggesting that *WUS* may function in the same pathway downstream of *CLV1*.

clv fas, *mgoun clv*, *stm mgoun*, *wus zll*, *stm zll* and *wus stm* double mutants are all largely additive suggesting that these genes act independently of each other (Leyser and Furner, 1992; Laux et al., 1996; Endrizzi et al., 1996; Laufs et al., 1998). *fas mgoun* double mutants have extremely reduced leaf and floral organ number (Laufs et al., 1998)

suggesting that these genes may function in partially redundant pathways to promote organogenesis on the flanks of the meristem.

The *TERMINAL FLOWER1* (*TFL1*) gene seems to be specifically involved in the maintenance of SAM fate. Mutations in the *TFL1* gene result in the eventual conversion of the SAM to an FM (Shannon and Meeks-Wagner, 1991; Alzarez et al., 1992; Shannon and Meeks-Wagner, 1993). The *TFL1* gene encodes a phophatidylethanolamine-binding protein that is expressed just below the SAM (Bradley et al., 1997). Based on expression patterns, *TFL1* does not appear to be a direct repressor of *LFY/AP1* in the SAM (see below) so *TFL1* may either act in a nonautonomous manner to inhibit floral identity or to promote SAM growth. For example, the SAM moves up from the incipient organ primordia by virtue of cell divisions just below the SAM, and *TFL1* might increase those cell divisions such that in a *tfl1* mutant floral primordia can overcome the SAM.

Genetic Dissection of Organ Initiation and Morphogenesis

When primordia are initiated on the flanks of the SAM, the primordia must gain an identity, leaf or branch or flower. Cells within the bulge must then interpret positional information, divide accordingly and contribute to the formation of a specific organ. Since little is known about the differentiation of leaves, flowers will be focused on.

The *PIN-FORMED* (*PIN*) and *PINOID* (*PID*) genes in *Arabidopsis* are required for the production of floral buds on the flanks of the SAM. In both *pin* and *pid* mutants, a fairly normal rosette of leaves is formed, then either no floral buds or only a few deformed floral buds are formed. The SAM often continues to grow resulting in a barren tip (Okada et al., 1991; Bennett et al., 1995). Though results so far are contradictory, it is thought that transport of the plant hormone auxin is required for floral bud initiation and the *pin/pid* mutants are defective in this process (Okada et al., 1991; Liu et al., 1993; Bennett et al., 1995).

Several mutations have been isolated where organ initiation occurs properly but the identity of the primordia is affected. In particular, mutations in several genes can cause the floral meristem to acquire SAM character. In *Arabidopsis*, mutations in the *LEAFY* (*LFY*) gene result in flowers that initiate floral organs in a spiral instead of a whorled pattern. In addition, the first whorl can contain leaf-like organs instead of sepals and the petals and stamen are reduced or missing. (Schultz and Haugh, 1991; Weigel et al., 1992; Huala and Sussex, 1992). Mutations in the *UNUSUAL FLORAL ORGANS* (*UFO*) gene resemble weak *lfy* alleles (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). *apetala1* (*ap1*) and *apetala2* (*ap2*) mutants can have leaf-like organs in place of sepals and a reduction in the number of petals. Secondary flowers can form in the axils of the first whorl leaves again suggesting that the flowers in these mutants have some SAM traits (Kunst et al., 1989; Bowman et al., 1989; Irish and Sussex, 1990; Bowman et al., 1993). Double mutant analysis has shown that *LFY, UFO*, *AP1* and *AP2* likely function in parallel to promote floral identity in the organs produced by the SAM (Weigel et al., 1992; Huala and Sussex, 1992; Schultz et al., 1993; Levin and Meyerowitz, 1995).

Consistent with their roles in promoting floral meristem identity, *LFY* encodes a novel protein and *AP1* encodes a MADS box transcription factor that are expressed on the flanks of the SAM and throughout the young floral primordia (Weigel et al., 1992; Mandel et al., 1992). *UFO*, on the other hand, encodes an F-box containing protein expressed in apical and floral meristems (Ingram et al., 1995; Lee et al., 1997). F-box proteins are known to interact with Skp1p and regulate the cell cycle (Bai et al., 1996; Ingram et al., 1997). Therefore, *UFO* may have a more direct role in patterning cell proliferation in developing meristems. *UFO* is expressed in the vegetative meristem but does not have a vegetative mutant phenotype (Levin and Meyerowitz, 1995; Lee et al., 1997) suggesting that it might have a redundant function in early plant development.

Once a floral meristem is initiated, it then produces floral organs of specific identity in concentric whorls. Genetic analysis has revealed a number of homeotic genes, which act

in combination, to impart floral organ identity (Bowman et al., 1991). Some of these genes, like *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), appear to specifically control floral organ identity (Bowman et al., 1989, 1991), whereas several of the genes have additional roles. For example, the *AP1* and *AP2* genes discussed above as controlling floral meristem identity also control sepal and petal identity in the flower (Kunst et al., 1989, Bowman et al., 1989, Irish and Sussex, 1990). The *AGAMOUS* (*AG*) gene, which controls stamen and carpel identity, also controls cell proliferation in the center of the floral meristem (Bowman et al., 1989).

AP1, *AP3*, *PI* and *AG* all encode MADS (MCM1, AGAMOUS, DEFICIENS, SRF) box transcription factors that are expressed in the whorls in which they act (Yanofsky et al., 1990; Mandel et al., 1992; Jack et al., 1992; Goto and Meyerowitz, 1994). Exactly how the floral homeotic genes function alone and in combination to direct organ identity is unknown; however, a gene with sequence similarity to the petunia *NAM* gene appears to be a direct target of *AP3* regulation. Transgenic experiments suggest that the *AP3*-regulated *NAM*-like gene might be involved in the switch between cell division and cell expansion as modes of organ growth (Sablowski and Meyerowitz, 1998).

The *CLV* genes, which regulate cell proliferation in the SAM, likely play a similar role in the FM. Plants homozygous for *clv1* or *clv3* mutations have floral meristems which become enlarged shortly after they are initiated on the flanks of the SAM (Clark et al., 1993, 1995). Extra floral organs are formed, especially stamen and carpels (Figure 2 A-B), and the usually determinate flower can maintain a central meristem that can continue to proliferate and form a fifth and sixth whorl of carpels. This suggests that the *CLV* genes regulate floral meristem cell proliferation and are required to cease meristematic growth after the production of carpels. The supernumery floral organs are likely the result of the enlarged floral meristem suggesting that there is a spacing mechanism that determines where organs can form.

Several other genes identified so far also control cell proliferation within regions of the floral meristem. *AG* has a role in floral meristem determinacy that can be separated from its role in organ identity (Sieburth et al., 1995). *AG* is expressed in the third and fourth whorls of the developing flower (Yanofsky et al., 1990; Drews et al., 1991) and targeted misexpression of *AG* shows that it is required in the fourth whorl to cease meristematic growth (Jack et al., 1997). *CLV1* is also necessary to limit cell proliferation in the meristem, but *CLV1* expression disappears before carpels are initiated (Clark et al., 1997) suggesting that *CLV1* plays an early role to establish floral determinacy. Mutations in the *SUPERMAN* (*SUP*) gene result in flowers with extra stamens and reduced carpels (Schultz et al., 1991; Bowman et al., 1992). *SUP* encodes a Zn-finger transcription factor expressed in the adaxial half of the stamen primordia suggesting that *SUP* functions cell autonomously to limit third whorl proliferation and non-autonomously to promote fourth whorl proliferation (Sakai et al., 1995). Double mutant analysis suggests that these genes function independently to pattern cell divisions in the fourth whorl (Bowman et al., 1992; Clark et al., 1993).

How are site of organ initiation determined? Floral organs are formed by increased periclinal cell divisions in specific regions of the floral meristem (Meyerowitz, 1997). There are at least two ways in which the number of organ initiation sites can be affected. As mentioned for *clv*, an increase in the size of the meristem would provide more room for organs to form. Also, there may also be a chemical signal that patterns organ initiation sites that could be modulated directly. Two *Arabidopsis* mutants, called *perianthia* (*pan*) and *wiggum* (*wig*), have a very similar increase in the number of sepals and petal (Running and Meyerowitz, 1996; Running et al., in press). However, *wig* mutants have an increase in the width of the floral meristem at the time of sepal and petal initiation, whereas *pan* mutants have a wild-type looking floral meristem. Therefore, the *PAN* gene is a good candidate for being directly involved in cells interpreting their position and dividing/initiating organs accordingly.

Morphogenesis is less well understood. Once a flower or an organ is initiated, it must undergo a complex series of coordinated cell divisions, cell expansions and cell death to make a mature organ. Only a few candidate genes have been described so far that are specifically involved in organ morphogenesis. *tousled* (*tsl*) mutants have fewer floral organs than wild type and the organs are deformed (Roe et al., 1993, 1997a). Similarly, the *ETTIN* gene plays a role in both organ number determination and morphogenesis of the carpels (Sessions and Zambryski, 1995). The *ERECTA* (*ER*) gene likely plays a more general role in the expansion of meristem derived tissues as *er* mutants have shorter stems and siliques although the numbers and patterning of organs is normal (Torii et al., 1996).

Molecular models for the positioning and morphogenesis of floral organs have been more elusive. *ETTIN* and *PAN* are both required to limit the number of floral organ initiation sites. *ETTIN* encodes a DNA-binding protein similar in sequence to transcription factors that bind auxin-responsive elements in plant promoters (Sessions et al., 1997). *PAN* encodes a bZIP transcription factor (Running, Williams, Chuang and Meyerowitz, unpublished data). Both genes are expressed throughout the SAM and young FM. They also have specific patterns of expression later in floral organs. *TSL* encodes a nuclear localized ser/thr protein kinase that is expressed throughout many tissues/organs (Roe et al., 1993, 1997a, 1997b) so it may play a general role to promote organogenesis/ morphogenesis. The *ER* gene encodes a putative receptor-kinase similar in sequence to *CLV1* (Torii et al., 1996). *ER* is expressed throughout the SAM and young FM, as well as in developing petals, stamen and carpels and may be involved in cell elongation (Robert W. Williams and Elliot M. Meyerowitz, unpublished).

One aspect of FM development that cannot readily be studied in *Arabidopsis* is adaxial/abaxial patterning (akin to dorsal/ventral patterning in animals). Many plant species, but not *Arabidopsis*, have zygomorphic flowers; that is the floral organs have a different size or shape around the axis of the flower. In *Antirrhinum*, the upper (or adaxial) petals are bigger than the abaxial petals. The *CYCLOIDEA* (*CYC*) and *DICHOTOMOUS* (*DICH*)

genes are involved in determining adaxial fate. Mutants in either gene cause a partial conversion of adaxial organs towards an abaxial fate. The *cyc dich* double mutant has a complete conversion. These genes are homologous to each other but they do not contain any recognizable motifs. The *CYC* gene is expressed in the adaxial region of the developing FM suggesting that it directly controls adaxial cell fate (Lou et al., 1996). Some of the organs that are affected in the *cyc* mutants are not contained within the *CYC* expression domain suggesting a nonautonomous action. *cyc* and *dich* mutants also have an organ number increase similar to *pan* mutant in *Arabidopsis* so *CYC* and *DICH* may also have a role in patterning organ initiation sites.

Questions of Biological Specificity

Many of the genes identified so far that control meristem patterning and growth are members of gene families. For example, there are many homeobox genes in the *Arabidopsis* genome. Based on the arrangement of motifs and on the homeodomain sequence, the plant homeobox genes are divided into several subfamilies. There are two *Knox* (*Kn1*-like homeobox) gene classes. Class I *Knox* genes, including *Kn1* and *STM*, have nearly identical homeodomain sequences and are generally expressed within subdomains of the SAM and FM (Kerstetter et al., 1994). The class II *Knox* genes resemble each other more than they resemble *Kn1* and are expressed in many tissues other than just the SAM or FM (Kerstetter et al., 1994). There are other homeobox gene families, like the *Arabidopsis GL2* which encodes a homeodomain-leucine zipper protein involved in root epidermis patterning and leaf trichome morphogenesis (Rerie et al., 1994), that are more distantly related to *Kn1*.

Are the biological functions of the plant homeobox genes directed by intrinsic DNA binding specificity alone, or are there other determinants? In vitro binding sites have only been determined for a single class I KNOX protein (Krusell et al., 1997), but all of the

class I *Knox* genes encode nearly identical residues at the positions thought to confer binding specificity of the animal homeodomain proteins (Gehring et al., 1994). Ectopic expression studies show that different *Knox* genes under control of the same promoter can result in different phenotypes (Matsuoka et al., 1993; Lincoln et al., 1994; Chuck et al., 1996; Williams, 1998), so the *Knox* genes have distinct roles. Either small amino acid changes in or near the homeodomain can alter DNA binding specificity, other domains of the KNOX proteins or cofactors can modulate the DNA binding specificity or the DNA binding sequence is not crucial to its' function.

More is known about the function of the MADS box transcription factors. With the exception of *AP2*, all of the known floral homeotic proteins have MADS domains. The *Arabidopsis* genome also encodes many other MADS box proteins of unknown function. *In vitro* studies have shown that MADS proteins bind DNA as dimers and most bind very similar DNA sequences. However, only certain hetero- or homodimers can actually bind DNA (Riechmann et al., 1996b). Plant overexpressing different MADS box genes have specific phenotypes showing that even though they have very similar DNA binding specificities, they function differently *in vivo* (Krizek and Meyerowitz, 1996b; Mizukami and Ma, 1992). Analysis of chimeric genes suggests that the region after the MADS domain largely controls the overexpression phenotype (Krizek and Meyerowitz, 1996a). Altering the DNA binding specificity of the MADS domain does not alter the biological function (Riechmann and Meyerowitz, 1997). Therefore, there must be cofactors that modulate the function of the MADS domain proteins.

The CLV1 and ER receptor-kinases are also members of a large gene family. This family is characterized by the presence of extracellular leucine-rich repeats. A putative steroid receptor in *Arabidopsis* also encodes an LRR-kinase similar to *CLV1* (Li and Chory, 1997). Large scale sequencing projects and low stringency hybridization have uncovered numerous more LRR-kinases in the *Arabidopsis* genome. Of the ones analyzed

so far, they seem to multimerize and autophosphorylate (Horn and Walker, 1994; Williams et al., 1997).

Sequence data and expression analysis seem to group the LRR-kinases into two subfamilies. The most closely related gene to *CLV1* is a gene called *CLAVATA1-LIKE 4* (*CLL4*). Whereas *CLV1* is expressed in the central region of the SAM and FMs, *CLL4* is expressed on the flanks of the SAM and FMs in what appears to be a complementary pattern to *CLV1* (Williams and Meyerowitz, unpublished data). So this family may have acquired roles in meristem function. *RLK5* and *CLAVATA1-LIKE 2* (*CLL2*) are express in the abscission zone and tapetum respectively (Williams and Meyerowitz, unpublished data). Therefore, members of this subfamily may have assumed roles in regulating cell death in developing organs/tissues.

CLV1, RLK5 and several other non-LRR-kinase have been shown to interact with KAPP (Stone et al., 1994, Braun et al., 1997; Williams et al., 1997), so KAPP likely regulates many different receptor-mediated signaling pathways. However, nothing is known about the genes that function downstream of the LRR-kinase. Do they each interact with different effector molecules or is there a universal signaling cassette downstream of all the LRR-kinases? If they utilize the same down stream components then how is specificity built into the system?

Conclusions

How are patterns of cell division and cell expansion established and utilized to produce specific tissues? What are the signals that control organogenesis and confer organ identity? Plants provide an attractive system to address these questions because they rely solely on cell division/expansion during development and they maintain relatively simple and easily studied pools of undifferentiated cells throughout development, and cell death does not seem to occur in the SAM and young FM.

It is becoming clear that spatial and temporal patterns of gene expression, largely in response to positional cues, establish domains of identity, cell proliferation and cell expansion. Furthermore, some of the genes involved are beginning to be studied. However, *Arabidopsis* floral and meristem development is at a position where Drosophila development was at 8-10 years ago. Namely, a handful of transcription factors are known that regulate parts of the body plan but the signals and cellular interactions that establish domains of gene expression are not known and the targets of regulatory genes are just starting to be identified. However, with a few genes in hand, more specific questions can be asked and more specific screens can be devised.

In addition to addressing basic questions of plant development, the work that is beginning to unfold can have very interesting evolutionary implications. The last 60-70 million years have seen a tremendous radiation in floral morphology, and floral form is a direct consequence of meristem patterning and activity.

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Table 1. Summary of Genes

Gene	Mutant Phenotype	Homolog	Protein	Expression
STM	no SAM	maize Kn1	homeodomain	SAM/FM
WUS	defective SAM	?	?	?
ZLL	SAM terminates	?	novel	vascular/SAM
CUC2	no SAM, fused organs	petunia NAM	novel	ring around SAM
CLV1	enlarged SAM/FM	?	receptor-kinase	SAM/FM corpus
CLV2	enlarged SAM/FM	?	?	?
CLV3	enlarged SAM/FM	?	secreted	SAM/FM CZ
FAS1/2	enlarged SAM	?	?	?
MGOUN1/2	enlarged SAM	?	?	?
PIN	no flowers	?	?	?
PID	no flowers	?	?	?
LFY	FM to SAM	*Antirrhinum FLO*	novel	young FM
UFO	weak lfy	*Antirrhinum FIM*	F-box	SAM/FM
AP1	FM to SAM	*Antirrhinum SQA*	MADS box	young FM
AP2	FM to SAM	?	novel	wide*
TFL1	SAM to FM	*Antirrhinum CEN*	PEABP*	below SAM
AP3	homeotic	*Antirrhinum DEF*	MADS box	petal/stamen
PI	homeotic	*Antirrhinum GLO*	MADS box	petal/stamen
AG	homeotic/indeterminate	*Antirrhinum PLE*	MADS box	stamen/carpel
SUP	stamen proliferation	?	Zn-finger	third whorl
PAN	organ number	?	B-ZIP	young FM
WIG	organ number	?	?	?
TSL	organ number	?	protein kinase	wide
ETT	organ morphology	?	transcription	wide
ER	organ shape	?	receptor-kinase	wide

Summary of *Arabidopsis* genes discussed in the text. Mutant phenotype refers to defects in the SAM, young FM or mature flowers. Homologs refers to known genes in other plant species that have known mutant phenotypes and sequence similarity to the cognate Arabidopsis gene. A "?" means that either the gene has not been cloned, that no homolog has yet been identified and/or the expression pattern has not been determined. Due to space considerations, not all of the citations to the *Antirrhinum* homologs are listed in the references section.

* Gene is expressed in a wide and/or dynamic pattern,

* PEABP, phophatidylethanolamine-binding protein.

Figure 1. Structure of the SAM and FM. (A) A longitudinal section through an embryo showing the embryonic leaves (cotyledons), embryonic stem (hypocotyl), SAM and root meristem. (B) A longitudinal CLSM image of a SAM showing the characteristic cell layering. (C) A longitudinal section through an SAM and young flowers showing the approximate locations of the CZ and PZ. (D) A longitudinal section through a vegetative SAM with leaf primordia indicated. (E) A SEM of an inflorescence, or flowering SAM, showing several young floral buds formed on the flanks of the SAM. (F) A photograph of a plant showing many mature flowers. cots, cotyledons; hyp, hypocotyl; RM, root meristem, SAM, shoot apical meristem; FM, floral meristem; PZ, peripheral zone; CZ, central zone; LP, leaf primordia.

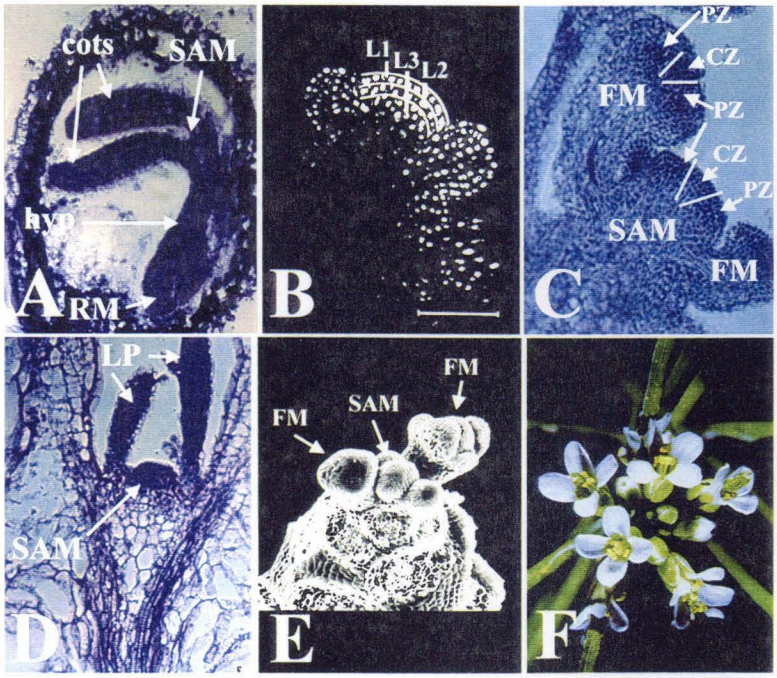
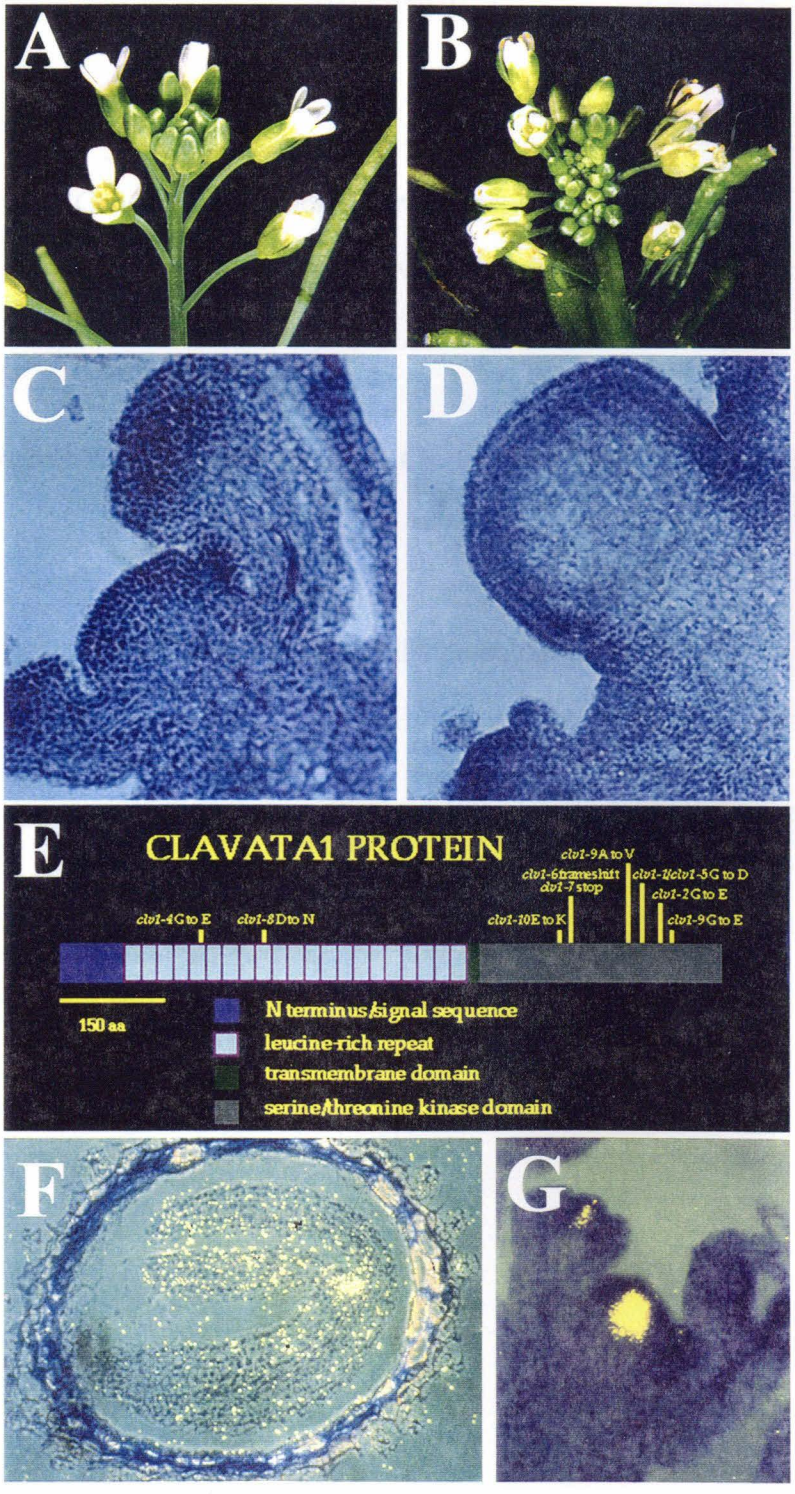

cots
SAM
hyp
RM
A
L1
L3
L2
B
PZ
CZ
FM
PZ
CZ
PZ
SAM
FM
C
LP
SAM
D
FM
SAM
FM
E
F

*Figure 2.*The *CLAVATA1* gene. (A) An inflorescence from a wild-type plant. (B) An inflorescence from a *clv1-8* homozygote. (C) Section through a wild-type inflorescence (same section as in Figure 1C). (D) A section through an inflorescence from a *clv1-4* homozygote. The photos taken in (C) and (D) are at the same magnification. (E) Schematic representation of the deduced CLV1 amino acid sequence with the location and molecular lesion found in 10 *clv1* alleles. (F) Expression of the CLV1 gene in a mature embryo. (G) Expression of CLV1 in an inflorescence SAM and a young floral meristem. In (F) and (G), the signal is seen as yellow grains.


A
B
C
D
E
CLAVATA1 PROTEIN
clv1-9A to V
clv1-6frameshift
clv1-7 stop
clv1-1/clv1-5G to D
clv1-2G to E
clv1-4G to E
clv1-8D to N
clv1-10E to K
clv1-9G to E
150 aa
N terminus/signal sequence
leucine-rich repeat
transmembrane domain
serine/threonine kinase domain
F
G

CHAPTER 1

Genetic and Physical Characterization of a Region of *Arabidopsis* Chromosome 1 Containing the *CLAVATA1* Gene

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*These two authors contributed equally to this work.

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The new nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF049870.

Abstract

With the increasing use of *Arabidopsis* as a model system for understanding plant genetics, development and biochemistry, a detailed description of the genome is necessary. As such, focused projects are underway to map and sequence the *Arabidopsis* nuclear genome. In the course of cloning *CLAVATA1* (*CLV1*) (Clark et al., 1997), we have characterized the surrounding region on chromosome 1. High resolution mapping crosses were done and four restriction fragment length polymorphism (RFLP) clones were mapped relative to a *clv1* allele. A yeast artificial chromosome (YAC) contig, spanning greater than 700 kb, was constructed. Three cosmids spanning the *CLV1* locus were analyzed and approximately 24 kb of genomic DNA was sequenced, including a continuous stretch of 18 kb. In addition to generating clones in this region of chromosome 1, we have analyzed the size, spacing and organization of several contiguous genes.

Mapping the *CLV1* Locus

The *CLV1* gene, which encodes a receptor-like kinase (Clark et al., 1997), was previously mapped to the bottom of chromosome 1 (Koornneef et al., 1983). To generate meiotic recombination breakpoints near a *clv1* allele, two mapping crosses were performed (Figure 1). Double mutants were first constructed between *clv1-1* and the flanking visible markers *ap1-1* and *ga2*. Like *clv1-1*, the *ap1-1* and *ga2* mutants are both in the Landsberg-*erecta* (L-*er*) ecotype. Both double mutants, *clv1-1 ap1-1* and *clv1-1 ga2*, were independently crossed to wild-type plants of the Columbia (Col-0) ecotype. The resulting F_1 progeny were allowed to self-fertilize. By selecting F_2 individuals that were homozygous for only a single mutation (i.e., *clv1-1* or *ap1-1* single mutants), we selected plants containing meiotic recombination breakpoint in the regions adjacent to *clv1-1* (see Figure 1). In the 10 cM region between *clv1-1* and *ap1-1*, a total of 124 recombinant progeny were identified. In the 8 cM region between *clv1-1* and *ga2*, 82 recombinant progeny were scored. This provided a mapping resolution of approximately one breakpoint per 0.1 cM or 20 kb on either side of *clv1-1*, based on a ratio of 200 kb/cM (Chang et al., 1988).

Each recombinant F_2 plant was allowed to self-fertilize, and genomic DNA was isolated from each F_3 family separately. Genomic clones known to be polymorphic between the L-*er* and Col-0 ecotypes were then mapped by Southern blot analysis of F_3 genomic DNA (Chang et al., 1988; Nam et al., 1989). A total of four RFLP clones were mapped relative to *clv1-1*. N7-24 and m532 mapped 19/124 and 1/124 recombinants proximal to *clv1-1*, respectively. m237 and g6838 both mapped 9/82 recombinants distal to *clv1-1* (Figure 2). This fine scale mapping revealed that the order of the RFLP clones and their positions relative to *clv1-1* are different than previously reported (Hauge et al., 1993). Only one of the 124 recombination events between *clv1-1* and *ap1-1* was shown to lie between m532 and *clv1-1*. Thus m532 lies approximately 20 kb away from *clv1-1*.

Construction of the YAC Contig

To construct a physical map of the *CLV1* region, three different RFLP clones were hybridized to the yUP, EW and EG YAC libraries (Guzman and Ecker, 1988; Ward and Jen, 1990; Grill and Somerville, 1991). The N7-24 clone hybridized to yUP3G5, yUP7C8, yUP17F5, EW4E10 and EW6G11 (Figure 2). The restriction pattern of N7-24 hybridizing fragments was altered in the yUP7C8 clone so it was determined that N7-24 lies at one end of the YAC insert. yUP7C8 did not hybridize to a nearby proximal marker, KG-24, so yUP7C8 probably extends towards the *CLV1* gene (data not shown). Clone m532 hybridized to yUP4B3, yUP7C7, yUP13C10, and EG8D6. Clone g6838 also hybridized to yUP4B3 and EG8D6 as well as to EW1G5. yUP7C7 hybridized to m532 and m237 but not to g6838. This indicates that even though m237 and g6838 mapped the same distance from *clv1-1* based on recombinants, g6838 is distal to m237.

The left and/or right ends of some of the YACs were isolated by either inverse PCR or plasmid rescue (Putterill et al., 1993; Schmidt et al., 1992) (Table 1). The left end of yUP13C10 hybridized to both yUP7C8 and yUP3G5, indicating that the region from markers N7-24 to m532 was contiguous (Figure 2). The left end of yUP4B3 was used to isolate a lambda genomic clone called B2. A polymorphism between L-*er* and Col-0 was identified and B2 was mapped distal to g6838. B2 was also used to isolate two more YACs, EW11G6, and EW16B12. The right end of EW11G6 was used to isolate two more YACs, yUP17A11 and yUP23G3. Neither yUP17A11 or yUP23G3 hybridized to the left end of EW11G6 so they presumably extend distally.

With the exceptions of EW16B12, yUP17A11 and yUP23G3, the size of each YAC clone was determined by pulsed field gel electrophoresis. Using the YAC sizes and some additional hybridization data summarized in Table 1, a complete physical map of the *CLV1* region was obtained (Figure 2). Based on N7-24 being approximately 2.3cM

from g6838 and the two markers being at least 490kb apart, this gives an estimate of 170kb/cM in this region.

Fine Scale Mapping and Sequencing of the *CLV1* Region

The YAC clone EG8D6 was used as a probe to isolate 31 Col-0 derived cosmid genomic clones (Olszewski et al., 1988) in the region spanning *clv1-1*. m532 was used to screen this pool of genomic clones, and two cosmids called cosQ and cosY were purified. Both cosmids contained the same *Eco*RI polymorphism as m532 and both mapped 1/124 recombinants proximal to *clv1-1*. Insert DNA from cosY and cosQ was used to isolate two more hybridizing clones, cosR and cosT, respectively. cosR mapped 3/124 recombinants proximal to *clv1-1* and cosT mapped 1/82 distal to *clv1-1* (Figure 3A). Therefore, the overlapping cosQ and cosT clones contained the *CLV1* locus.

Both cosQ and cosT were digested with *Hin*d III and each fragment was cloned into the pGEM 7zf vector (Promega). Most of the fragments were sequenced in their entirety. To generate a complete *Hin*d III map of the two cosmids, primers were designed 100 to 200 bases from each *Hin*d III site complementary to the sequence generated from the Universal or Reverse primers. Every primer combination was used in PCR reactions with intact cosQ or cosT as the template. Primer combinations that gave PCR products of the appropriate size (approximately 300 to 400 bp) represented adjacent *Hin*d III fragments. The identity of each PCR product was verified by sequencing. The largest continuous stretch of genomic sequence is 18 kb (Figure 3B).

Each *Hin*d III fragment from cosQ and cosT as well as some of the *Hin*d III fragments from cosY were used to screen a flower cDNA library (Weigel et al., 1992). Each positive cDNA clone was converted to plasmid and sequenced using a combination of Universal, Reverse and internal primers. A total of eight different genes were identified in the approximately 40 kb region.

Sequence Analysis of Transcription Units

Three of the eight genes have previously been identified: ß-tubulin, an antifungal gene called *PDF1.1* (Penninckx et al., 1996) and *ARAC5*. There are at least nine ß-tubulin genes in the *Arabidopsis* genome (Snustad et al., 1992); the one identified here is ß-tubulin 1 (Oppenheimer et al., 1988). While this ß-tubulin gene is preferentially expressed in roots, it was readily detected in the flower/inflorescence cDNA library. The *ARAC5* gene was a direct submission to Genbank (accession number U52350). It encodes a small GTP binding protein of the Rho family. Rho proteins have been speculated to play a role in transducing signals directly to the actin cytoskeleton. The gene with the most similar sequence to *ARAC5* is a *rho* gene from garden pea (*Pisum sativum* L.) called *Rho1Ps* (Yang and Watson, 1993). Even though the function of these plant Rho proteins is not known, it is likely that the *Rho1Ps* and *ARAC5* genes may play very similar roles in pea and *Arabidopsis*.

Of the remaining five genes, four had sequence motifs or sequence similarities that suggest possible functions. A zinc-finger containing gene, called *AtZF1* (corresponding to EST R30294), lies on cosY(Figure 3). The gene encodes a protein of at least 462 amino acids that contains a Cys-2/His-2 type zinc-finger in the middle of the ORF. We also isolated a cDNA clone, of unknown map location, called *AtZF2*, that is 80% identical and 88% similar to *AtZF1* (corresponding to EST R65207) over a 192 amino acid region.

Immediately distal to ß-tubulin there is a thaumatin-like gene, termed *AtTL1*. Thaumatins, and osmotins, are a class of secreted proteins that are part of the plants' response to stress and/or pathogens. The gene with closest sequence similarity to *AtTL1* is an *Arabidopsis* gene called *PR5K*, which contains an extracellular thaumatin domain and

a cytoplasmic serine/threonine protein kinase domain (Wang et al., 1996). Based on the genomic sequence, the AtTL1 protein does not have a kinase domain.

The *CLV1* gene, which is just distal to *AtTL1*, is completely contained on cosT and partially contained on cosQ. It encodes a putative receptor-kinase, with an extracellular domain comprised of 21 leucine-rich repeats. Further analysis of this gene has been presented elsewhere (Clark et al., 1997).

Distal to *PDF1.1* there is a gene encoding a protein with sequence similarity to the yeast vacuolar sorting protein VPS35 (Paravicini et al., 1992). *VPS35* is required in yeast for proper targeting of a subset of vacuolar proteins. The *Arabidopsis* gene is called *AtVPS-35* (Clark et al., 1997).

In between *AtTL1* and *CLV1* lies a gene that encodes a small, novel protein. This gene, called *orfI-1*, has an open reading frame of 141 amino acids and contains a putative signal sequence. This protein may be secreted. The remainder of the deduced amino acid sequence has no recognized motifs, but the protein is largely hydrophobic.

Genomic Organization of the Genes and Intergenic Regions

The cDNA and genomic sequences were compared for five of the genes: ß-tubulin 1, *AtTL1*, *orfI-1*, *CLV1* and *PDF1.1*. All five genes are contained in the 18 kb of continuous genomic sequence discussed above. The ß-tubulin gene contains two introns, one of 794 bp and the other of 88 bp. The *AtTL1* gene contains an 80 bp intron after the putative signal sequence and *PDF1.1* contains a 107 bp intron following the signal sequence. The *CLV1* gene contains a 79 base intron in a conserved position in many other serine/threonine kinase genes (Clark et al., 1997; Chang et al., 1992). All the intron splice sites conform to the consensus for *Arabidopsis* (A/C)AG | GTAAGT(A/T) - (C/T)AG | G(A/T). Furthermore, the codon usage of these five genes closely follows that of a variety of other *Arabidopsis* genes (http://www.dna.affrc.go.jp/~nakamura/CUTG.html).

Four of the five ORFs are closely spaced, with 585 bp between ß-tubulin 1 and *AtTL1* and 2 kb between the *AtTL1* gene and *orfI-1*. There is a larger 5.7 kb gap between *CLV1* and *PDF1.1*; however, located between these two genes is a 104 bp region with sequence similarity to *PDF1.1* that is probably the result of a duplication event. There is a stop codon in the *PDF1.1* pseudo-gene, so it likely does not represent a functional gene.

Analysis of the nucleotide content of coding versus non-coding sequences indicates that coding sequences are noticeably more GC rich than non-coding regions. On average, intergenic and intron sequences are 60-80% AT whereas coding regions are only 40-60% AT. By plotting %AT content of the 18 kb region with a 50 base window size, the ORFs are easily distinguished as regions where the AT content drops below 60% (data not shown).

Significance

The data reported here confirm several predictions about the *Arabidopsis* genome. First, we estimate that there is 170 kb/cM in the region analyzed. This is similar to previous estimates of 150-200 kb/cM (Chang et al., 1988). The density of genes observed in this study, which corresponds to approximately 20,000 genes per haploid genome, is consistent with previous estimates for *Arabidopsis* and other angiosperms (Meyerowitz, 1994; Bevan et al., 1998). In this region of the genome, the splice site sequences and codon bias closely resemble those for a number of other *Arabidopsis* genes. It is interesting to note that two of the genes, *AtTL1* and *PDF1.1*, contain introns immediately following a potential signal sequence.

In addition to reporting the sequence and exact map positions of eight genes, the data presented have several additional uses. First, the continuous genomic sequence matches up with four BAC end sequences in the TIGR database (T16B16TRD, F05H06Sp6, T19O22TF and T20K17TR/T20M12TR) thus mapping those BAC clones to

chromosome 1. In addition, the molecular clones generated here could be used as entry points for positional cloning of other genes that map near *CLV1*. For example, *EMB25*, *EMBRG3*, *PIN*, *IAR1*, *EMB54*, *EMB17*, *EMB120*, *BOT1*, *EMB156*, and *SUS2* all map within 10 cM of *CLV1* (D. Meinke, http://mutant.lse.okstate.edu/).

Finally, as many different plant species have vastly different genome sizes but have regions where the genetic maps are similar (Shields, 1993), these data may be used to identify the cognate homologue of any gene in this region from other flowering plant species. This is particularly useful since many plant genes, including *CLV1* (Clark et al., 1997) and *AtZF1*, appear to be members of gene families. Identifying the orthologue of a gene in another plant species can be difficult but may be aided by knowing which genes map near the gene of interest.

Materials and Methods

Plant Growth and Procedures

Seeds were sown on a 1:1:1 mixture of soil:perlite:vermiculite saturated with dilute Gnatrol (Abbott Laboratories) and Daconil fungicide (Chevron Ortho). Seeds were imbibed on soil at 4° C for four days then transferred to 25° C with 600 ft. candles of continuous light. Plants were fertilized once a week with Plantex 20-20-20 (Plantco) and sprayed as needed with Orthene insecticide (Chevron Ortho). Crosses were set up by emasculating the female parent and then hand pollinating with the male parent.

Total genomic DNA was isolated by grinding 2g of plant tissue in 0.2M Tris (pH8), 0.1M EDTA, 1% N-lauroylsarcosine, 100ug/ml proteinase K and then ethanol precipitated twice. The final nucleic acid pellet was re suspended in 4 ml of 1g/ml CsCl with 1mg ethidium bromide and centrifuged overnight at 53K rpm. The DNA band was

pulled and the ethidium bromide was extracted with SSPE saturated isopropanol. The amount of DNA was quantitated by agarose gel electrophoresis.

RFLP Mapping and Library Screening

RFLP markers were mapped by digesting 3 µg of genomic DNA with an appropriate restriction enzyme. DNA was then separated by agarose gel electrophoresis and transferred to a nylon membrane (Sambrook et al. 1989). The RFLP probe was labeled using the Boehringer-Mannheim random prime labeling kit following the manufacturer's instructions. The Southern blot was hybridized overnight at 65° C in 6X SSPE, 5X Denhardt's reagent, 0.5% SDS and 100 ug/ml salmon sperm DNA. The filters were washed at 65° C in 0.1X SSPE/0.1% SDS. YAC libraries, cosmid libraries and cDNA libraries were screened using the same conditions as above. The cosmid library was provided by Neil Olszewski (Olszewski et al. 1988). A flower cDNA library packaged in lambda-ZAP (Weigel et al. 1992) was also screened.

YAC Isolation and Analysis

The Ecker (yUP; Guzman and Ecker 1988), Ward (EW; Ward and Jen 1990) and Sommerville (EG; Grill and Somerville 1991) YAC libraries were used. Yeast genomic DNA was isolated by growing 50 ml cultures in YPD for two days at 30° C. Cells were centrifuged and resuspended in 2.5 ml of SE (1.5M sorbitol, 2 mM EDTA pH 8, 10 mM Tris pH 7.4). Spheroplasts were made by adding 3 µl ß-ME and 10 µl of a 100 mg/ml yeast lytic enzyme solution (ICN) and incubated at 37° for 30 minutes. Spheroplasts were diluted with 15 ml of SE, spun down then resuspended in 1.5 ml of 5X TE. Cells were lysed by adding 150 µl of 10% SDS and incubating at 65° for 30 minutes. Cell debris was removed by adding 660 µl of 5M K-acetate and then centrifuged, 10K for 10

minutes. The supernatant was transferred to a new tube, and the nucleic acids were precipitated with isopropanol. The pellet was resuspended in TE + RNase A and incubated at 37° for 20 minutes. The solution was then extracted with phenol/chloroform twice, and with chloroform once. DNA was precipitated with ethanol and resuspended in 150 µl of TE. YAC ends were isolated essentially as described by Putterill et al. (1993) and Schmidt et al. (1992). Briefly, YAC left ends were isolated by plasmid rescue using either *Xho* I or *Nde* I. YAC right ends were isolated by iPCR using the C and D primers (Schmidt et al. 1992).

The size of individual YAC clones was determined by PFGE. Intact yeast chromosomes were isolated by: creating spheroplasts in 0.5% LMP agarose and incubated overnight at 37°. The agarose plugs were washed in 0.5 M EDTA pH 9.5 and then incubated overnight at 50° in 0.5 M EDTA pH 9.5, 1% sarkosyl and 1 mg/ml proteinase K. The plugs were washed and stored in 50 mM EDTA pH 8. Undigested chromosome preperations were analyzed by pulse-field gel electrophoresis. The gels were visualized by ethidium bromide staining, blotted to nylon filters, and probed with a plasmid clone containing yeast CEN4 sequences.

Genomic and cDNA Sequencing

*Hin*d III restriction maps of cosmids were generated and each fragment was cloned into the *Hin*d I site of the pGEM 7zf vector (Promega). Each cloned fragment was sequenced using the USB Sequenase 2.0 kit. Oligonucleotides based on the generated sequence were designed from both strands 100 to 200 bases into each fragment. Each combination of primers was used in PCR reactions with intact cosmid DNA as the template. If two primers represented adjacent *Hin*d III fragments, then a fragment of predictable size would be amplified. The resulting PCR fragments were cloned into the TA cloning vector (Invitrogen) and sequenced to verify their identity. The remainder of each cloned

*Hin*d III fragment was sequenced by successive rounds of primer generation and sequencing (primer crawling). Lambda-ZAP cDNA clones were converted to pBluescript based plasmids following the manufacturer's instructions. The cDNA clones were sequenced using the USB Sequenase 2.0 kit.

Sequence Analysis

ORF identification, translations and nucleic acid alignments were done using the MacVector program version 4.1.4 (Kodak IBI). Computer sequence analysis of the gene products was accomplished using the Wisconsin GCG package on a VAX computer. Specifically, protein databases were searched using the BLAST program, protein motifs were identified using the MOTIF program, and alignments were done using the BESTFIT program.

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Table 1. Characteristics of yeast artificial chromosomes. Each YAC is listed with the restriction enzyme(s) that was used to generate the plasmid based left end (L.E.) probes and the restriction enzyme and primer, in conjunction with primer C69 (Schmidt et al., 1992), used to generate the inverse PCR based right end (R.E.) specific probes. B2 and S3 are lambda genomic clones that correspond to the yUP4B3 left end and the yUP7C8 right end, respectively. The size of each YAC clone is given as well as the names of the clones that hybridize to it. The clones listed in italics in the "Hybridizes" column do not hybridize to the specified YAC. HE, *Hinc*II/*Eco*RV double digest.

YAC	L.E.	R.E.	Size(kb)	Hybridizes
yUP3G5	X/N[1]	all	310	N7-24, *S3, 6G11le*
yUP7C8	*Nde*I	HE70[2] (S3)	280	N724, *6G11le*
yUP17F5	X/N	*Alu*71 HE70	100	N724, S3 *6G11le*
EW4E10	X/N	*Alu*71	120	N724, KG24, S3 *6G11le*
EW6G11	*Nde*I	*Alu*71	120	N724 *KG24, S3*
EW11G6	*Xho*I	all	110	B2, 16B12le
EW16B12	*Xho*I	all	n.d.	B2
yUP17A11	X/N	*Alu*	n.d.	11G6re
yUP23G3	X/N	all	n.d.	11G6re
yUP4B3	*Xho*I (B2)	-	320	532, 6838, B2 *16B12le*
EW1G5	*Xho*I	-	120	6838 *B2*
EG8D6	*Xho*I	all	180	532, 6838, *B2*
yUP13C10	*Xho*I	all	320	532 *6838, B2*
yUP7C7	*Xho*I	HE70	230	237, 532 *6838, B2*

[1] "X/N" indicates that XhoI and NdeI derived left end plasmid rescue clones were generated, 2 "HE" indicates that a *Hinc*II/*Eco*RV double digest was used to generate the iPCR right end fragments.

Table 2. List of identified open reading frames as well as any homology or sequence motifs that indicate a function.

Gene	Homology	Function	GenBank Accession
AtZF1	zinc finger	transcription factor?	R30294
ß-tubulin 1	*ß-tubulin*	cytoskeleton	M20405
AtTL1	*PR5K*	stress/pathogen perception?	AF049870
orfI-1	novel/secreted?	unknown	AF049870
CLV1	receptor-kinase	meristem development	U96879
PDF1.1	cysteine-rich	pathogen defense	Z27258
AtVPS-35	*VPS35*	vacuolar sorting	U96877/U96878
ARAC5	GTP-binding	signal transduction	U52350

Figure 1. Crossing strategy for fine scale mapping of RFLP clones near the *CLV1* gene. In both crosses, the solid black chromosomes are derived from the L-*er* ecotype and the stippled chromosomes are derived from the Col-0 ecotype. Only the relevant F_2 recombinants are shown. *ga2* single mutants were not isolated due to their germination defects in the absence of exogenously applied gibberellic acid. Chromosomes and loci are not drawn to scale.

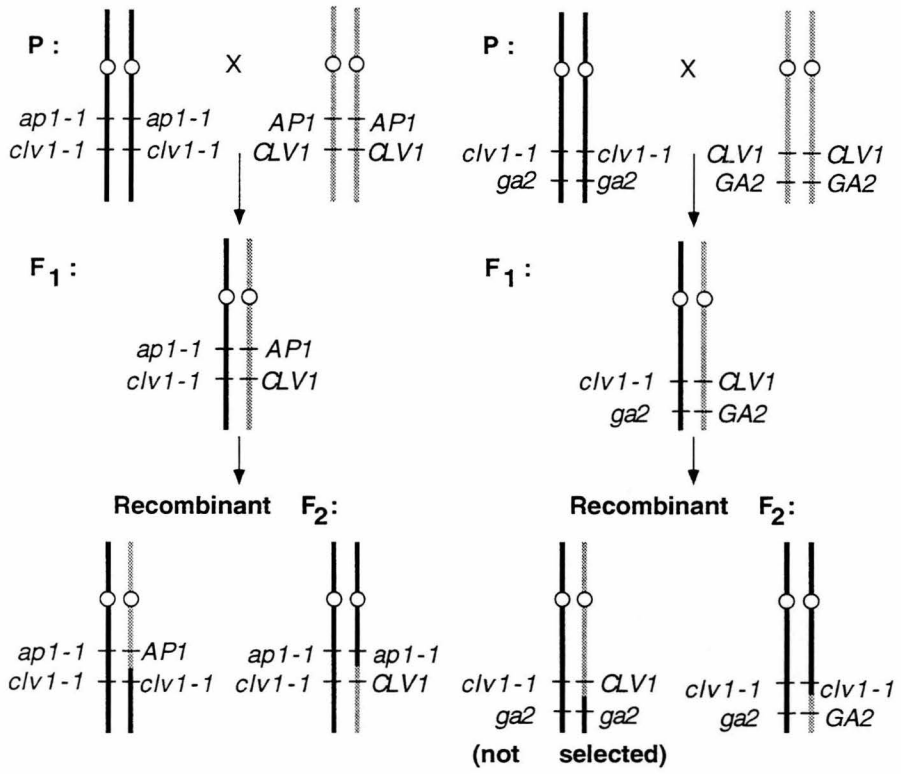
P :
X
ap1-1
clv1-1
ap1-1
clv1-1
AP1
CLV1
AP1
CLV1
F1 :
ap1-1
clv1-1
AP1
CLV1
Recombinant F2:
ap1-1
clv1-1
AP1
clv1-1
ap1-1
clv1-1
ap1-1
CLV1
P :
X
clv1-1
ga2
clv1-1
ga2
CLV1
GA2
CLV1
GA2
F1 :
clv1-1
ga2
CLV1
GA2
Recombinant F2:
clv1-1
ga2
CLV1
ga2
(not selected)
clv1-1
ga2
clv1-1
GA2

Figure 1

Figure 2. Genetic and physical map of the *CLV1* region. The number of recombinants is listed for the mapped molecular clones.

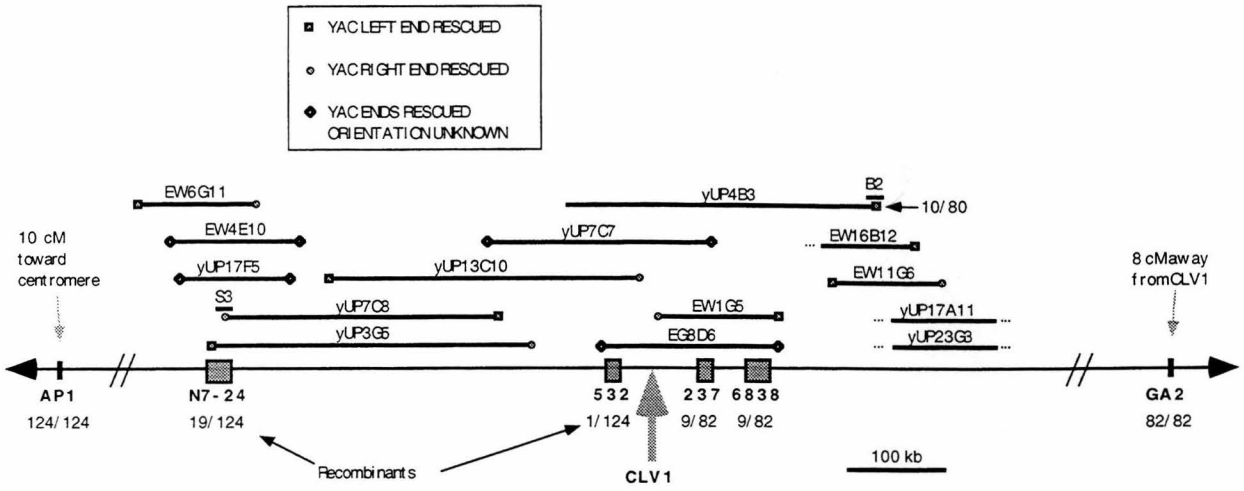
YAC LEFT END RESCUED
YAC RIGHT END RESCUED
YAC ENDS RESCUED
ORIENTATION UNKNOWN
EW6G11
EW4E10
yUP17F5
S3
yUP7C8
yUP3G5
yUP4B3
B2
10/80
yUP7C7
EW16B12
yUP13C10
EW11G6
EW1G5
EG8D6
yUP17A11
yUP23G8
10 cM
toward
centromere
8 cMaway
fromCLV1
AP1
124/124
N7-24
19/124
532
1/124
237
9/82
6838
9/82
GA2
82/82
Recombinants
CLV1
100 kb

Figure 2

Figure 3. Fine scale mapping and sequencing in the *CLV1* region. (A) The four mapped cosmids are listed with the number of recombinants. (B) The *Hin*dIII restriction map and locations and direction of transcription of each ORF is given for most of cosQ and cosT and part of cosY.

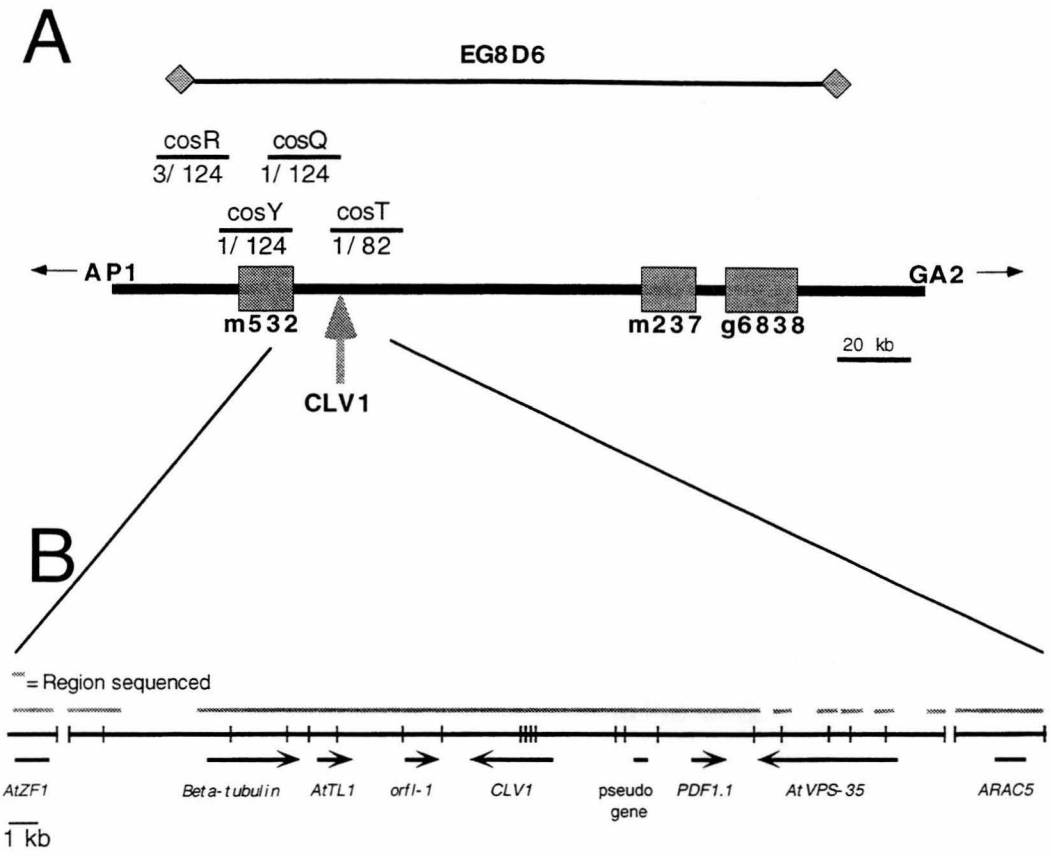
A
EG8D6
cosR
3/ 124
cosQ
1/ 124
cosY
1/ 124
cosT
1/ 82
AP1
GA2
m532
m237
g6838
20 kb
CLV1
B
= Region sequenced
AtZF1
Beta-tubulin
AtTL1
orf1-1
CLV1
pseudo gene
PDF1.1
AtVPS-35
ARAC5
1 kb

Figure 3

CHAPTER 2

The *CLAVATA1* Gene Encodes a Putative Receptor Kinase That Controls Shoot and Floral Meristem Size in *Arabidopsis*

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*These two authors contributed equally to this work.

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The accession numbers for the sequences reported in this article are U96879 for *CLV1* and U96877 and U96878 and for *AtVPS-35*.

Summary

The shoot apical meristem is responsible for above-ground organ initiation in higher plants, accomplishing continuous organogenesis by maintaining a pool of undifferentiated cells and directing descendant cells toward organ formation and differentiation. Normally, proliferation and differentiation are tightly balanced, so that the structure and size of the shoot meristem is maintained. However, Arabidopsis plants homozygous for mutations at the *CLAVATA1* (*CLV1*) locus accumulate massive numbers of undifferentiated cells. We describe the molecular cloning and expression pattern of the *CLV1* gene. It encodes a putative receptor-kinase, suggesting a role in signal transduction. The extracellular domain is composed of 21 tandem leucine-rich repeats (LRRs) that are similar to LRRs found in animal hormone receptors, such as thyroid-stimulating hormone receptor and follicle-stimulating hormone receptor. We provide evidence that *CLV1* expression in the inflorescence is specifically associated with meristematic activity.

Introduction

In higher plants, organogenesis is not limited to embryonic development but occurs throughout the life of the plant. This is accomplished by initiating and maintaining meristems, groups of stem cells that have the ability to divide and replenish themselves as well as to produce progeny that can enter specific differentiation pathways. During embryogenesis, only a simple body plan is established with a basal root meristem and a shoot meristem. After germination, the shoot meristem grows to produce leaves, axillary meristems and floral meristems in succession. For proper formation of an adult plant, the shoot meristem must maintain a tight balance of initiating organs while retaining undifferentiated cells. As the shoot tip grows away from the undifferentiated cells as a result of mitosis, the newly distal cells enter a specific developmental pathway leading to eventual differentiation (Figure 1A). These features account for the continued maintenance of the shoot structure and for its function in organogenesis.

The angiosperm shoot meristem has traditionally been divided into two regions. The central zone (CZ) is characterized by slowly dividing cells and is surrounded by the peripheral zone (PZ) where rates of mitosis increase (Steeves and Sussex, 1989; Lyndon, 1990). Some evidence indicates that the PZ is where organ initiation occurs, but it remains unclear if the CZ corresponds to the undifferentiated cells of the shoot meristem (Steeves and Sussex, 1989). The shoot meristem in most dicotyledonous flowering plants, including Arabidopsis, is also composed of three cell layers (Figure 1C). The tunica consists of the L1 and L2 cell layers that remain clonally distinct, due to anticlinal cell divisions, and give rise to epidermis and mesophyll respectively. The corpus, or L3, lies beneath the tunica layers and is characterized by more variable cell division planes and cell size. The corpus produces pith and vascular tissue. The fact that the three cell layers are largely clonally isolated but are coordinated in their growth and organ initiation indicates that cell-cell signaling may be important in shoot meristem function (Meyerowitz, 1997).

In Arabidopsis, the shoot meristem produces floral meristems which give rise to the floral organs (sepals, petals, stamens and carpels) in a whorled pattern (Smyth et al., 1990). The floral meristem is similar in structure to the shoot meristem but has a different fate. In addition to initiating different types of organs and in a different phyllotactic pattern than the shoot meristem, the floral meristem is a determinate structure: in the flower, carpels are initated in the center of the flower meristem, terminating growth; while the shoot meristem maintains undifferentiated cells and can initiate an indeterminate number of flower anlagen. So the balance of keeping undifferentiated cells while initiating organs is altered in flowers as compared to shoots.

Many mutants with defects in shoot and/or floral meristem development have been isolated in Arabidopsis. A few exhibit phenotypes specific to meristem development. Mutations in one such gene, *CLAVATA1* (*CLV1*), result in plants with enlarged shoot and floral meristems (Figure 1B; Leyser and Furner, 1992; Clark et al., 1993). In the shoot meristem, the *clv1* phenotype is apparent as early as in the mature embryo (Running et al., 1995). As the *clv1* mutant plants continue through vegetative and inflorescence development, their shoot meristems continue to enlarge by accumulation of undifferentiated cells. In contrast, wild-type meristems are maintained at nearly the same size throughout the plants' life (Leyser and Furner, 1992; Clark et al., 1993). This overproliferation can result in a *clv1* shoot meristem growing over 1000-fold larger (by volume) than wild type. A separate gene, *CLAVATA3* (*CLV3*), has mutant phenotypes identical to those of *clv1* mutants, and appears to function in the same pathway as *CLV1* (Clark et al., 1995).

clv (*clv1* or *clv3*) mutations have a similar effect on the development of the floral meristem as on the development of the shoot meristem (Figure 1; Clark et al., 1993; Clark et al., 1995). The *clv* floral meristem can be twice as tall as the wild-type floral meristem at the earliest stage of organ initiation. Analysis of maturing flowers revealed that the *clv* floral meristem continues to proliferate and give rise to a large mass of undifferentiated cells in the center of the flower, instead of terminating like the wild-type floral meristem. Thus,

in both the shoot and the floral meristem, *clv* mutations affect the balance of cell proliferation versus cell differentiation.

In this report we describe the cloning and sequence of the *CLV1* gene. Its coding sequence suggests that CLV1 may function as a signal transduction component that acts in the communication of cell division and/or differentiation signals. We also present evidence that in the inflorescence meristem *CLV1* mRNA is expressed specifically in a central region of the shoot and early flower meristems, implying nonautonomous action in the surface cell layer(s) of the meristem.

Results

Cloning *CLV1*

We isolated the *CLV1* gene based on its map position. *CLV1* maps on chromosome 1, between the gibberellin biosynthesis gene *GA2* and the floral homeotic gene *AP1* (Koornneef et al., 1983). It was found that 1 of 124 recombination events between *ap1-1* and *clv1-1* lies between the RFLP clone m532 (Chang et al, 1988) and *clv1-1* indicating that m532 maps approximately 0.1cM (roughly 17kb) proximal to *CLV1*. Using m532 as a probe, we isolated two overlapping cosmids (Olszewski et al., 1988), cosQ and cosT, that contain *CLV1*. CosQ contains the same polymorphism as m532. 1 of 82 recombinants between *clv1-1* and *ga2* lies in cosT, delimiting the *CLV1* gene to the region covered by the cosmids. CosT, but not cosQ, restored a wild-type phenotype when transformed into *clv1-4* mutant plants, and thus contained the whole *CLV1* gene.

CosT was subcloned and sequenced (Figure 2), which revealed four genes, each with similarity to sequences in the database. These included a leucine-rich repeat-kinase (LRR-kinase), the plant defensin gene *PDF1.1* (Penninckx et al. 1996), a gene we call *AtVPS-35* that is similar to a yeast vacuolar sorting protein (VPS-35; Paravicini et al., 1992) and

ARAC5 , a recently identified small GTP-binding protein (Figure 2). To further define the region that corresponds to *CLV1*, we mapped the nearest recombination breakpoint on the distal side of *CLV1*. This breakpoint mapped to a single fragment in the middle of the cosmid, ruling out the *ARAC5* and *AtVPS-35* genes. The remaining region contained the LRR-kinase and *PDF1.1*.

We sequenced the *PDF1.1* gene from plants homozygous for three different *clv1* alleles (*clv1-1*, *clv1-5* and *clv1-4*) and found no changes relative to the wild-type sequence. In sequencing the putative LRR-kinase, we identified either a missense or a frame-shift mutation in each of the seven *clv1* alleles that we analyzed (see below and Table 1).

To confirm that the putative LRR-kinase corresponds to the *CLV1* gene, we transformed *clv1-1* mutants with constructs containing various regions of genomic sequence spanning the *CLV1* gene. The CLV1 6.3 construct provided consistent, partial suppression of the *clv1-1* phenotype (Figure 3A). Transformation of the CLV1 5.7 construct provided no rescue of the mutant phenotype whereas the CLV1 5.1 construct provided partial rescue of the *clv1-1* phenotype, suggesting that there may be a 3' regulatory sequence or a negative regulatory element in the 5' region in between the XbaI and the SacI sites (Figure 3B).

CLV1 Sequence

The nucleotide and deduced amino acid sequence of *CLV1* is shown in Figure 4A. The transcriptional start site was determined by 5′ RACE. The poly-A site of the transcript was determined by sequencing the 3′ end of a cDNA clone. A canonical poly-A addition site (AATAAA; Joshi, 1987) was found 28 nucleotides upstream of the poly-A tail. Based on the cDNA 5′ RACE and 3′ poly-A site, the *CLV1* transcript is predicted to be 3262 bases long. This is consistent with RNA blot data that shows an approximately 3.4 kb mRNA

(data not shown). The *CLV1* genomic sequence contains a single 79 nucleotide intron at a position that is conserved in the similar LRR-kinase gene *TMK1* (Chang et al., 1992).

The amino terminus of the CLV1 protein sequence has a potential signal peptide that directs secretion. This is followed by a putative extracellular domain consisting of 21 complete LRRs (Figure 4B) with 15 N-linked glycosylation consensus sites (N-X-S/T). As with many LRR-containing proteins, the LRR region is flanked by pairs of conservatively spaced cysteines. The LRR domain is followed by a stop-transfer sequence, suggesting a transmembrane domain. The putative intracellular domain contains all of the conserved residues found among serine/threonine protein kinases (Figure 4A) and recombinant CLV1 protein has protein kinase activity (RWW, SEC and EMM, unpublished data).

The *CLV1* gene was amplified and sequenced from DNA isolated from plants homozygous for seven different *clv1* mutant alleles (Table 1). In each sequence a single substitution relative to wild-type was uncovered. Two of the strong mutant alleles, *clv1-4* and *clv1-8*, contained missense mutations in the LRR domain. For the five other alleles, all weak to intermediate in phenotype, we identified lesions in the kinase domain. Four of the alleles, *clv1-1*, *clv1-2*, *clv1-5* and *clv1-9*, all contain missense mutations. Interestingly, one of the weakest known alleles, *clv1-6*, contains an insertion of an A near the beginning of the kinase domain that would theoretically replace most of the kinase domain with 16 novel amino acids.

CLV1 is similar in sequence to a number of other genes coding for LRR-kinases. The most closely related complete gene in the database is *RLK5* (Walker, 1993), an LRR-kinase of unknown function in Arabidopsis. CLV1 and RLK5 are 37% identical and 58% similar at the amino acid level over their entire length. The kinase domains are 47% identical and the LRR domains are 36% identical at the amino acid level. The majority of the identical residues in the putative receptor domain are at conserved LRR positions; at non-conserved positions, the CLV1 and RLK5 LRR domains are only 22% identical. Other

similar Arabidopsis genes include *TMK1* (Chang et al., 1992), *TMKL1* (Valon et al., 1993) and the *ERECTA* gene, which is required for proper internode elongation (Torii et al., 1996). A non-Arabidopsis plant gene of identical structure to *CLV1* and *RLK5* is *Xa21* (Song et al., 1995). *Xa21*was isolated from rice based on its ability to provide resistance to the bacterium *Xanthomonas oryzae* pv. *oryzae* race 6.

In addition to the known proteins, there are several Arabidopsis expressed sequence tags (ESTs) that are similar to regions of the CLV1 protein. These include LRR-containing sequences and kinase domain sequences. Some of these sequences are more similar to CLV1 than RLK5 is to CLV1, indicating that the Arabidopsis genome contains many *CLV1*-like genes.

Phylogenetic Analysis

A number of plant kinases, many of which are involved in development and disease resistance pathways, have been isolated over the past several years. To determine the relationship of these kinases as well as their relationships to kinase domains found in animals, we performed a phylogenetic analysis using a heuristic tree-building program. After calculating a single shortest tree using no weighting for similar amino acids, this tree was used as the starting point for a heuristic analysis using a matrix that weighted amino acids changes based on the similarity of the altered residue. A single shortest tree was produced and six other trees, shorter than the starting tree, were also identified. The strict consensus of the seven trees is shown in Figure 4D.

Several relationships are supported in the consensus tree. First, all the plant kinases analyzed, except for CTR1, form a separate lineage distinct from the animal kinases. Most of the S-receptor-like kinases cluster together, but the same is not true for the LRR-kinases. The kinases involved in disease resistance, Pto and Xa21, do not form a distinct group.

Pto, which has a kinase but no extracellular domain, belongs to an LRR-kinase group, including CLV1, RLK5 and ERECTA.

CLV1 Expression Patterns

Since we know so little about how and where receptors of any type function in plants, we sought to establish the pattern of *CLV1* mRNA expression. To avoid cross-hybridization to closely related members of the LRR-kinase gene family, we defined a *CLV1*-specific probe. The region coding for the carboxy-terminus of the kinase domain (the last 300 nucleotides of the ORF) showed little sequence homology to known kinases and the corresponding probe hybridized only to *CLV1* in a low-stringency genomic DNA blot hybridization experiment (data not shown). ^{35}S-labeled sense and antisense riboprobes were generated from this fragment and were hybridized to sections of wild-type Arabidopsis inflorescence tissue (see Methods).

In the shoot meristem, *CLV1* was expressed in a patch of cells across the center of the meristem. It was not expressed in the L1 layer, as can be seen from both longitudinal serial sections (Figure 5B-F) and transverse sections (data not shown). *CLV1* signal was not detected on the flanks of the shoot meristem in cells that are presumably flower anlagen. In the most central region, *CLV1* may also be expressed in the L2 layer. Sense controls reveal that this signal was specific for *CLV1* and not due to background (Figure 5G-I).

In the floral meristem, *CLV1* expression seemingly changes preceding organ initiation. *CLV1* expression is down-regulated in the floral anlagen, and no expression is detected in stage 1 flowers (Figure 5B-D, all stages according to Smyth et al., 1990). In stage 2 floral meristems, signal was detected. This was observed in a central region, but was absent from the presumed sepal anlagen (Figure 6D). Further down-regulation preceded organ initiation. By stage 4, *CLV1* expression was absent from whorl 2 and at least the outer portion of whorl 3 in the petal and stamen anlagen (Figure 6A-C,E). By stage 5, *CLV1*

expression was only detected in the very center of the floral meristem (Figure 6A-C) and no signal was detected after this stage (Figure 6F). As in the shoot meristem, *CLV1* mRNA was not detected in the L1 layer of the flower meristem, and we were unable to determine if it is expressed in the L2 layer cells.

Discussion

CLV1 as a Receptor-Kinase

We have cloned the Arabidopsis *CLV1* locus based on its map position, and sequence analysis indicates that it may function as a receptor-kinase. The presumed extracellular domain is composed almost exclusively of LRRs, which are known to be involved in protein-protein interactions. The crystal structure of the LRR-containing ribonuclease inhibitor (RI) complexed with RNase A has been solved (Kobe and Deisenhofer, 1995). Each repeat forms a section of ß-sheet and a section of α-helix and when combined with other repeats, the protein forms a donut-shaped structure with an inner face containing the parallel ß-sheets that contact the RNase A molecule, and an outer face containing the α-helix portions. Glycoprotein hormone receptors from animals, including luteinizing hormone receptor (LHR; McFarland et al., 1989), thyroid stimulating hormone receptor (TSHR; Parmentier et al., 1989), and follicle stimulating hormone receptor (FSHR; Sprengel et al., 1990), all contain extracellular LRRs that are responsible for high-affinity binding of their respective ligands. The repeat structure of this class of receptors has been compared to that of porcine RI (Jiang et al., 1995), and computer predictions suggest a common structure. CLV1 has a LRR repeat pattern very similar to that of the glycoprotein hormone receptors (Figure 4C). Based on this analysis, the strong *clv1-4* and *clv1-8* alleles result in amino acid substitutions in the ß-sheet face (*clv1-4* at position 4 and *clv1-8* at

position 1). Thus, the severe phenotype of these alleles might be due to reduced ligand binding.

The presumed intracellular domain coded by *CLV1* contains all of the conserved residues found among serine/threonine (ser/thr) protein kinases (Hanks and Quinn, 1991); furthermore, recombinant CLV1 protein has protein kinase activity (RWW, SEC and EMM; unpublished data). The most closely related sequences in the database are several Arabidopsis ESTs and the intracellular domains of RLK5 and TMK1, which have been shown to autophosphorylate on ser/thr residues in vitro (Chang et al., 1992; Horn and Walker, 1994). The three-dimensional structures of several protein kinases have been solved and shown to be similar to one another (De Bondt et al., 1993; Hu et al., 1994; Zhang et al., 1994). The location of the lesions in *clv1* mutants was analyzed based on the protein kinase A structure (PKA; Knighton et al., 1991). The amino acids altered in the weak/intermediate *clv1* alleles *clv1-1* (and the identical lesion in *clv1-5*), *clv1-2*, and *clv1-9* are all in residues that are conserved in PKA. For *clv1-1* and *clv1-9*, the altered residues lie in the activation loop, a region shown to be important in regulating kinase activity in a number of protein kinases (Johnson et al., 1996). The lesion in *clv1-2* is located 3' to the portion of the gene coding for the activation loop.

By analogy to animal receptor-tyrosine kinases, CLV1 could act to bind a ligand, leading to homodimerization. Previous genetic evidence has suggested that CLV1 acts as a multimer (Clark et al., 1995). Dimerization would bring the kinase domains in close proximity which may result in intermolecular phosphorylation. The nature of the *clv1-1* and *clv1-6* alleles also support such a model. The *clv1-1* mutation is semi-dominant (Clark et al., 1995) and results in an amino acid substitution in a conserved residue in the kinase activation loop. Thus, the clv1-1 protein may function in a dominant-negative manner by binding to and preventing intermolecular phosphorylation of a wild-type partner protein in heterozygous plants. Biochemical studies with RLK5 have demonstrated that the kinase domain can form dimers and transphosphorylate. Furthermore, an excess of catalytically

inactive RLK5 can inhibit the kinase activity of wild-type RLK5 protein (Horn and Walker, 1994). The *clv1-6* mutation, which results in a truncated protein with most of the kinase domain deleted, has a weak phenotype indicating that the kinase domain may be partially redundant with another protein, or that readthrough occurs.

After phosphorylation, the kinase domain would bind specific factors, analogous to SH2-containing proteins in animals, that would transmit the signal to downstream targets. One protein that could be involved in binding the phosphorylated CLV1 kinase domain is KAPP (Stone et al., 1994). KAPP contains a kinase-interaction (KI) domain linked to a type 2C protein phosphatase domain. The KI domain has been shown to associate with the phosphorylated kinase domain of RLK5, but not to a catalytically inactive form of the RLK5 kinase domain (Stone et al., 1994). The specificity of the KAPP-RLK5 interaction and the role of KAPP-binding in vivo are unknown.

CLV1 Signaling

If CLV1 acts as a receptor, the question arises as to what in vivo signal it responds. There are two potential models of CLV1 action - regulating cell division or cell differentiation - that could fit with CLV1 acting to perceive some signal. Cell division rates are tightly regulated in the shoot meristem; for example the cells in the central zone (CZ) divide much more slowly than those in the peripheral zone (PZ) (Steeves and Sussex, 1989; Lyndon, 1990). That the shoot meristem remains nearly the same size throughout the life of the plant indicates that the rate of division of central undifferentiated cells is closely balanced with the proportion of cells undergoing differentiation on the flanks. Organ-forming cells on the flanks are derived from the central undifferentiated cells, thus differences in cell division rates are presumably controlled based on positional cues. In *clv1* mutants, there is a gradual accumulation of undifferentiated stem cells. Because the pool of stem cells is increased by proliferation of these cells, and decreased by incorporation of these cells into organ

primordia, CLV1 could function either to repress cell division of the stem cells, or to promote their incorporation into organ primordia. Thus, CLV1 could act to perceive positional information regulating stem cell division rates. Alternatively, if CLV1 acts to promote the transition of cells reaching the flanks of the meristem toward organ formation and an eventual differentiated state, CLV1 could act to receive information regulating this transition. There are extra cells in the shoot meristem of the mature embryo in *clv1* mutants (Running et al., 1995), presumably before any differentiation has yet occured, thus adding support to the cell division model. However, the *CLV1* expression pattern could fit either of these models (see below).

If CLV1 represents one component of a novel signal transduction pathway, there must be a number of other components in the signaling pathway. Some could function specifically in the CLV1 pathway, while others may function in several signaling cascades. Candidates for other components of the CLV1 pathway include the gene products of *CLAVATA2* (CLV2; Koornneef et al., 1983), *CLV3* and *WUSCHEL* (*WUS*; Laux et al., 1996). Mutations at *CLV2* and *CLV3* have similar phenotypes as *clv1* mutants suggesting that these three genes could act on the same processes. In addition, we have previously demonstrated that CLV3 likely functions in the same pathway as CLV1 as the *clv1* and *clv3* mutants exhibit dominant interactions (Clark et al., 1995). Thus, CLV2 and/or CLV3 could be required for ligand generation or function as downstream components. Mutations at *WUS* exhibit phenotypes largely opposite those of *clv* mutants - failure to establish or maintain functioning stem cells in the shoot meristem, and reduced numbers of central organs/whorls in the flower meristem (Laux et al., 1996). Furthermore, *wus* mutations are epistatic to *clv1-4*. These data indicate that either CLV1 functions to negatively regulate WUS activity, or that WUS is required to establish the undifferentiated cells upon which CLV1 acts. Under the first hypothesis, WUS could be a target of CLV1 signaling.

CLV1 is similar in structure to Xa21, which confers resistance to *Xanthomonas oryzae* pv. *oryzae* race 6 in rice (Song et al., 1995). The tomato genes *Cf-9* and *Pto* are known to

be involved in pathogen recognition and defense and each have sequence similarity to various domains of the CLV1 protein (Cf-9 to the LRRs and Pto to the kinase; Martin et al., 1993; Jones et al., 1994). Thus, both disease resistance and developmental signaling share similar components suggesting a common origin for these pathways in plants. A similar observation has been made in animals. Components of the mammalian IL-1/NF-κB-mediated inflammatory response and the Drosophila Toll/Dorsal-mediated patterning pathways also share proteins with sequence similarity. Furthermore, proteins in the Drosophila Toll signal transduction pathway are used to activate antifungal genes in adult flies (Lemaitre et al., 1996). It is interesting to speculate that in two separate multicellular lineages, components of pre-existing regulatory pathways may have been recruited for pathogen defense, or that ancient environmental sensors have been recruited for use in cell-cell communication.

CLV1 Expression

In situ RNA hybridization experiments revealed that, in the inflorescence, *CLV1* is specifically expressed in the shoot and floral meristem. *CLV1* mRNA is found in the presumptive shoot meristem during embryo development, and is not detected in the root meristem (SEC and H. Sakai, unpublished data). *CLV1* is expressed in the shoot meristem in a region comprising a subset of the central stem cells and the inner portion of the organ forming region (based on expression of floral anlagen-specific genes such as *LEAFY* ; Weigel et al., 1992). This expression pattern fails to distinguish between the two main models for CLV1 action: regulating stem cell division or promoting the transition of cells reaching the flanks toward differentiation. *CLV1* is down-regulated on the outer edge of the organ-forming region and in the earliest flower primordia. Expression is reestablished in the flower meristem, so that by stage 2, expression is at a level comparable to that of the shoot meristem. In the flower meristem, the expression pattern is again consistent with

both models for *CLV1* action: regulating cell division rates in the center of the flower meristem or promoting the transition of flanking flower meristem cells toward organ primordia formation and differentiation.

That the expression of *CLV1* would be so tightly restricted was not expected a priori. Many receptors in a variety of systems are expressed ubiquitously, with their spatial regulation being conferred by the ligand. One possible reason for regulating *CLV1* expression might be to provide unidirectional signaling from a secreted ligand. If the information to which CLV1 is responding emanates from the flanks or the superficial cell layers, then limiting *CLV1* expression to a region interior to this signal would ensure that the signal is only perceived in a unidirectional fashion. Another possibility is that the presence of CLV1 in other cell types might interfere with other signaling pathways.

Another observation is that *CLV1* is not expressed in the L1 layer of the shoot and floral meristems. In *clv1* mutants, undifferentiated cells accumulate in all layers of the shoot and flower meristem. This indicates that CLV1 has a cell-nonautonomous function. Several explanations are possible for the mechanism of the nonautonomous function. One could imagine, for example, that CLV1 mediates signaling from the L1 cells to deeper layers, allowing the clonally distinct but adjacent cells to coordinate their division and/or differentiation patterns. Induction between different cell layers requiring communication of this sort has been observed in plant development. Two notable findings include the specification of a nearly normal flower in Antirrhinum plants in which only the L3 layer contains a wild-type *FLORICAULA* gene (Hantke et al., 1995), and tomato plants whose floral meristem size appeared to be largely determined by the genotype of the L3 (Szymkowiak and Sussex, 1992). Alternatively, the CLV1 protein may be physically transported between cell layers. The maize Knotted-1 gene is transcribed in the corpus but the protein is found in the corpus and tunica (Smith et al., 1992; Jackson et al., 1994). The Knotted-1 protein has been shown to move through plasmodesmata in leaf mesophyll cells (Lucas et al., 1995). Similarly, the MADS box proteins DEFICIENS and GLOBOSA may

be transported between cell layers in the Antirrhinum floral meristem (Perbal et al., 1996). A similar process may account for CLV1 affecting L1 cell division and/or differentiation.

These examples of cell-cell communication of division and differentiation information in plants raise a more general possibility. Throughout plant development, the division and differentiation of adjacent cells must be closely coordinated. The absence of cell migration in plant development implies that the form of plant organs and the shape of meristems depends entirely on the numbers and patterns of cell divisions and cell expansion. In addition, plant organ structure depends on coordination of cell fates and cellular differentiation. To achieve the necessary coordination of the activities of cells, such as those in the L1 and L2, which are clonally only distantly related, requires a cell-cell communication mechanism for exchanging and/or relaying cell division or cellular differentiation information. The nature of the *CLV1* mutant phenotype and of the protein coded by the gene perhaps provides a single example of a general mechanism. The plant leucine-rich repeat receptor kinase family of genes is large, as indicated not only by the other genes already identified (in *Arabidopsis TMK1*, *RLK5*, and *ER* as well as *CLV1*), but possibly by additional partial cDNA sequences found in the Arabidopsis EST database. It could be that such receptor kinases play a general role in plants, with each layer or region of cells having its own family members. The general coordination of cellular activities in plant development may depend upon signaling from all cells to their neighbors via members of the LRR-kinase family. In this respect this family of receptors, found so far only in plants, may be functionally similar to the receptor-tyrosine kinases used so extensively in animals, but so far found only in animals.

Experimental Procedures

Plant Material

The *ap1-1*, *clv1-1* and *ga2* mutants are all in the Landsberg-*erecta* ecotype. The isolation of the *clv1-1* through *clv1-7* alleles have been previously described (Leyser and Furner, 1992; Clark et al., 1993; Koornneef et al., 1983). The *clv1-8* allele was originally called *fully fasciated* (Medford et al., 1992). The *clv1-9* allele was an EMS generated allele kindly provided by Steven Jacobsen.

Seeds were sown on a 1:1:1 mix of soil:perlite:vermiculite and imbibed for 3-5 days at 4° C. Plants were grown at 25° C under constant light. Plants were fertilized once a week and sprayed with insecticide as needed.

Cloning and Sequencing of *CLV1*

Meiotic recombination breakpoints were generated near *CLV1* by screening for recombinants between *clv1-1* mutants and the flanking markers *ap1-1* and *ga2*, when *clv1-1 ap1-1* and *clv1-1 ga2* double mutants in the L-*er* ecotype were crossed to wild-type Col-O plants. These recombinants were used to map RFLP clones relative to the *CLV1* gene by southern blot hybridization. m532 was used as a probe to isolate cosQ and cosT by colony hybridization. CosT was used to isolate cDNA clones from a flower library (Weigel et al., 1992). Each gene was PCR amplified and sequenced directly from seven *clv1* alleles. The 5' end of the *CLV1* transcript was defined by PCR-RACE using a kit (GibcoBRL) as outlined in the manufacturer's directions. The MacVector program version 4.1.4 by Kodak IBI was used to identify ORFs and nucleic acid alignments. The protein databases were searched using the BLAST program.

Rescue of *clv1* Mutant Phenotype

Regions of the *CLV1* genomic region were subcloned into the T-DNA containing vector pCGN1547 (McBride and Summerfelt, 1990) and transformed into *E.coli*. Recombinant

plasmids were identified by restriction enzyme analysis and then transformed into Agrobacterium tumefaciens strain ASE. Whole plants were then transformed using the vacuum infiltration method (Bechtold et al., 1993). Transgenic seedlings were selected by resistance to kanamycin and transplanted to soil. Transgenic plants were then grown and allowed to set seed as normal.

Phylogenetic Analysis

Amino acid sequences of all the kinase domains used for tree-building were aligned using the Clustal V program, and modified by hand. Regions sharing very little similarity among the kinase domains were removed from the sequences for phylogenetic analysis. The regions used for analysis correspond to residues 691-712, 716-723, 733-757, 762-781, 786-808, 813-841, 856-867 and 871-890 of CLV1. The aligned sequences were analyzed using the Paup 3.1.1 program using heuristic searches due to the large number of genes involved. We first started with a random tree for a heuristic analysis using amino acid identities (i.e., amino acid changes were of equal cost regardless of residue substituted). After 3 days of computation, the 8000 shortest trees were used for another heuristic search. A single shortest tree was generated from this analysis and was used as the starting tree for an heuristic search using a weighted matrix based on the BLOSUM 62 matrix (M. Frohlich, personal communication). The weighting takes into account amino acid similarities. Seven trees shorter than the starting tree were identified, although it should be noted that these trees are not shorter when using amino acid identities to calculate tree lengths. Bootstrapping was not performed because the large number of sequences involved meant that starting with a random tree, it took over 3 days of computation to identify the shortest tree.

In Situ Hybridization

A 330bp PCR product spanning the last 100 codons of the ORF and 30bp of 3'UTR hybridized to a single band on a genomic southern blot when washed at 50° C with 2XSSPE. This PCR product was cloned into the TA cloning vector (Invitrogen) to generate the pBW4KD4 plasmid. Antisense RNA probes were generated by linearizing pBW4KD4 with *Xba* I and synthesized with SP6 RNA polymerase. Sense control probes were synthesized using T7 RNA polymerase on pBW4KD4 linearized with *Hin*dIII. The remainder of the in situ procedure was done according to Drews et al. (1991) with modifications by Sakai et al. (1995).

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Table 1. *clv1* alleles

Allele	Lesion	Predicted affect	Phenotype	Reference
clv1-1	G –> A	Gly856 –> Asp	Intermediate	1, 2, 3
clv1-2	G –> A	Gly881 –> Glu	Intermediate	1
clv1-3	n. s.		Strong	1
clv1-4	G –> A	Gly201 –> Glu	Strong	2, 4
clv1-5	G –> A	Gly856 –> Asp	Intermediate	2
clv1-6	Frameshift	See Text	Weak	2
clv1-7	n. s.		Weak	2
clv1-8	G –> A	Asp295 –> Asn	Strong	5
clv1-9	C –> T	Ala839 –> Val	Weak	6

n .s. - not sequenced
References: 1, Leyser and Furner (1992); 2, Clark et al. (1993); 3, Koornneef et al. (1983); 4, McKelvie (1962); 5, Medford et al. (1992); 6, this work.

Figure 1. Diagram of wild-type and *clv1* shoot and flower meristems. Idealized longitudinal sections through inflorescences of wild type (A, C) and *clv1* (B). Undifferentiated stem cells (SC) and organ-forming regions (OF) of both the shoot meristem (SM) and flower meristem (FM) are shaded. In *clv1* mutants, this balance is disrupted, resulting in the accumulation of undifferentiated stem cells. 1, 2, 3 - stage 1, 2 and 3 flowers, respectively. (C) The clonally distinct L1, L2 and L3 cell layers of the tunica and corpus are indicated.

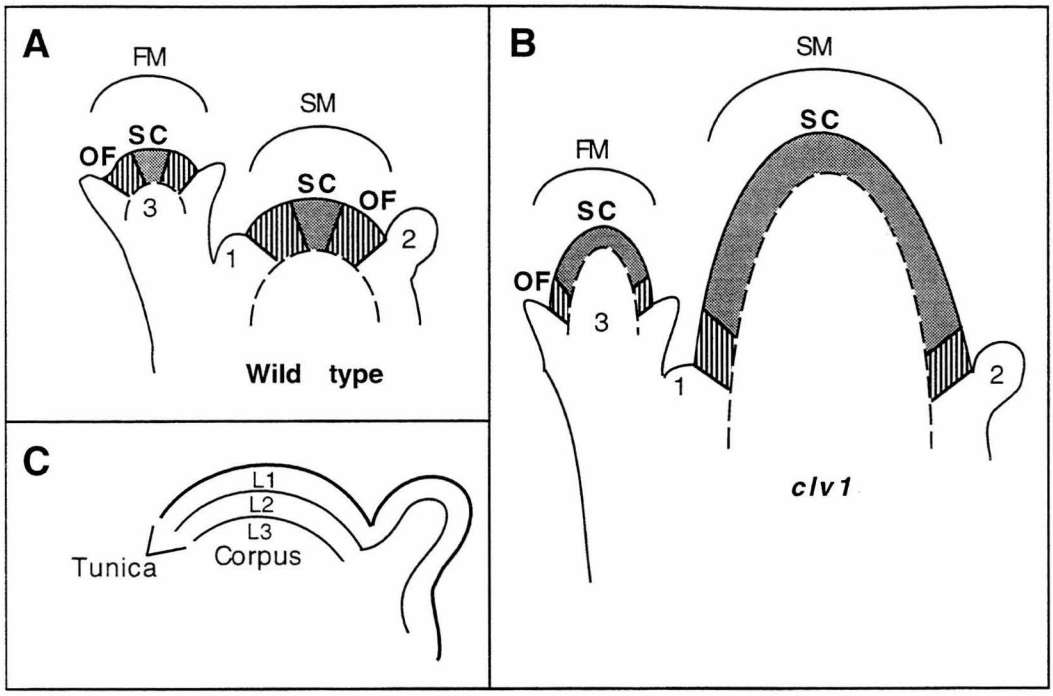

A
FM
SM
SC
OF
SC
OF
3
1
2
Wild type
B
SM
SC
FM
SC
OF
3
1
2
clv1
C
L1
L2
L3
Tunica
Corpus

Figure 2. Physical Map of Cosmid T. The location of each ORF and the direction of transcription is indicated by the arrows. The accession number for *ARAC5* is U52350. H, HindIII sites.

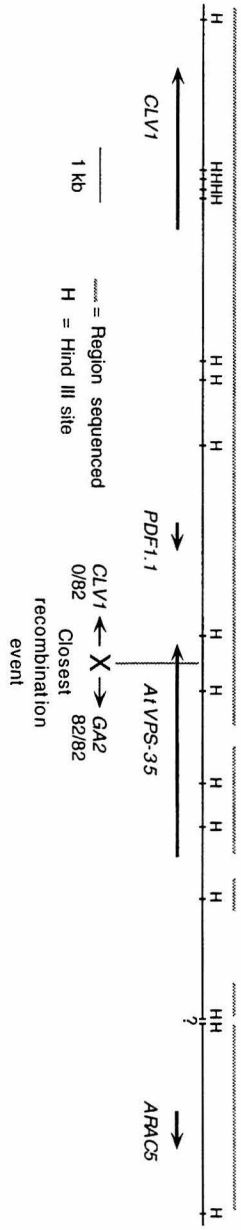
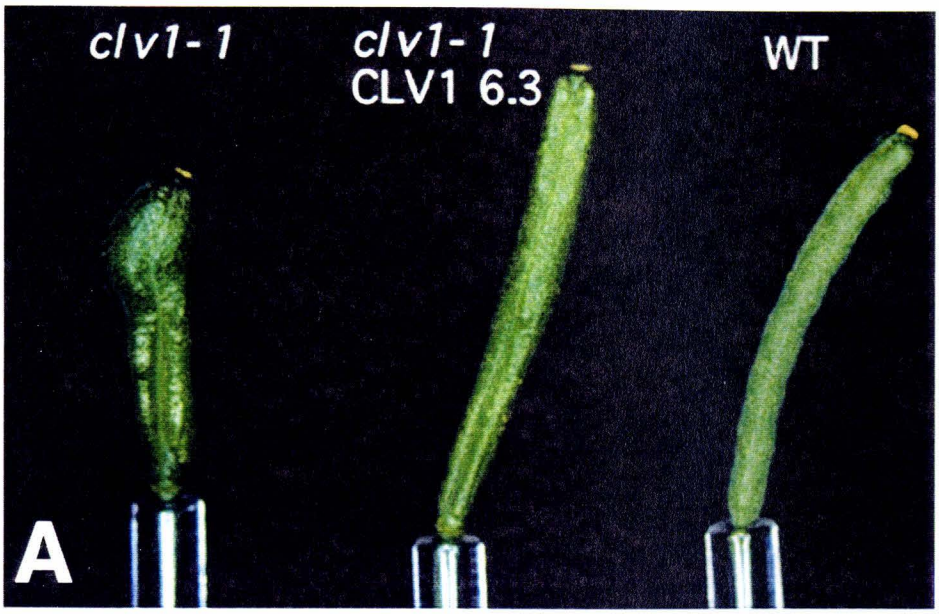
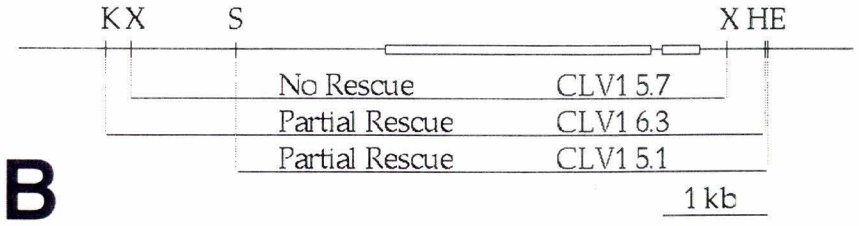

CLV1
PDF1.1
AtVPS-35
ARAC5
1 kb
= Region sequenced
H = Hind III site
CLV1
0/82
GA2
82/82
Closest
recombination
event
?

Figure 3. CLV1 6.3 genomic construct provided partial suppression of the *clv1-1* phenotype. (A) A comparison of typical gyneocia from a *clv1-1* plant, a *clv1-1* plant transformed with the CLV1 6.3 construct, and a wild-type plant. (B) A diagram of the *CLV1* genomic region indicating the sequences used to attempt rescue of the *clv1-1* mutant phenotype. E, EcoRI. K, KpnI. X, XbaI. S, SacI. H, HindIII. The HindIII site on the right end of the map in (B) corresponds to the HindIII site at the far left end of the map shown in Figure 2. The internal HindIII sites are not shown.


clv1-1
clv1-1
CLV1 6.3
WT
A


K X
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X H E
No Rescue
CLV1 5.7
Partial Rescue
CLV1 6.3
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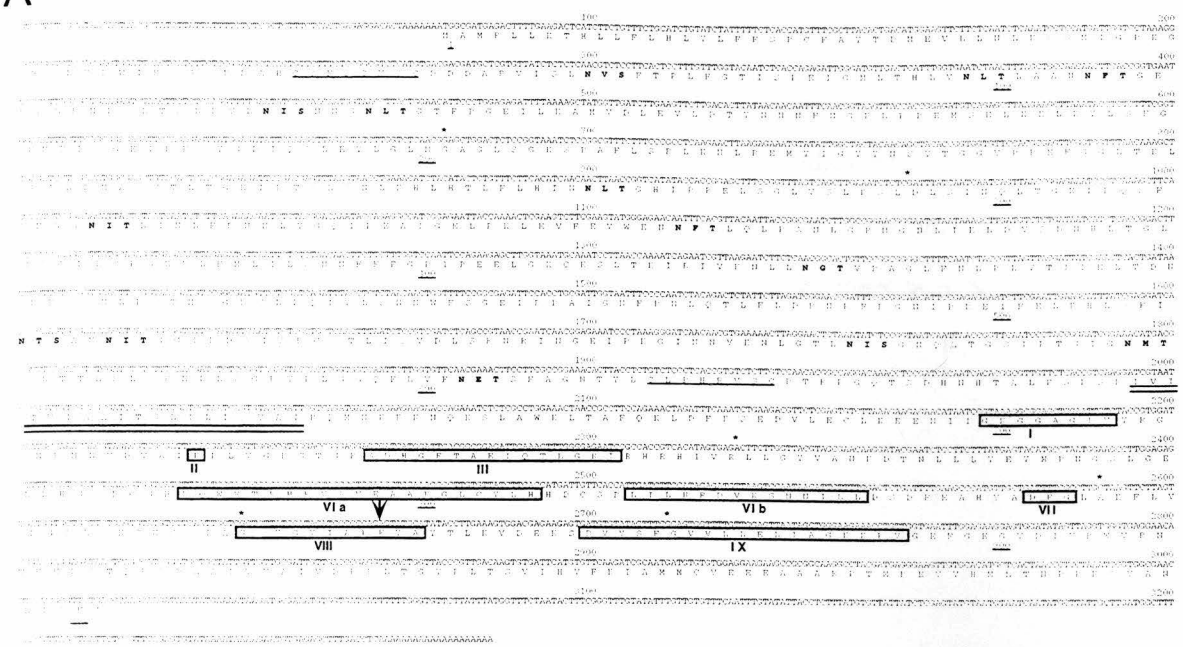
CLV1 5.1
1 kb
B

Figure 4. The *CLV1* gene and protein sequence. (A) DNA sequence corresponding to the *CLV1* transcript, with the predicted amino acid sequence shown below. The nucleotide numbering is listed above the nucleotide sequence, and the amino acid numbering (underlined) is given below the protein sequence. The paired cysteines flanking the LRR region are underlined and potential N-linked glycosylation sites in the extracellular domain are in bold lettering. The predicted transmembrane sequence is double underlined and flanked by charged residues. In the kinase domain, the conserved regions are boxed and indicated with roman numerals, following the nomenclature of Hanks and Quinn (1991). The arrowhead indicates the position of the intron and the asterisks indicated the positions of the mutations listed in Table 1. (B) The LRR domain of CLV1 is presented, with the 21 complete and 2 partial repeats aligned. (C) The CLV1 LRR consensus (bottom) is compared to the animal hormone receptor repeat structures (top). X indicates any amino acid, other capital letters indicate consensus amino acids (Φ = hydrophobic), and lower case letters indicate residues frequent at a position. Aspartic acid or glutamic acid is present at position 1 in 8 of 21 CLV1 repeats and 7 of 21 animal repeats (only those repeats analyzed in Jiang et al. (1995) were included in this calculation). Asparagine is present in 16/21 animal repeats at position 5 or 6. Proline is present in 11/21 animal repeats at position 12 or 13. The region of each repeat predicted to be in a ß-sheet (E), loop (L) or alpha-helical (H) conformation is indicated below the consensus alignments. (D), Phylogenetic relationships between plant and animal kinase domains showing the consensus of the seven shortest trees. The kinase sequences used in the analysis were: CLV1 (this article), RLK1, RLK4, RLK5 (Walker, 1993), TMK1 (Chang et al., 1992), ER (Torii et al., 1996) and CTR1 (Kieber et al., 1993) from Arabidopsis; SRK (Stein et al., 1991) from Brassica napus, PTO (Martin et al., 1993) and FEN (Loh and Martin, 1995) from tomato; Xa21 (Song et al., 1995) from rice; ZMPK1 (Walker, 1993) and CR4 (Becraft et al., 1996) from maize; DROR (Wilson et al., 1993), TWICHIN and SCR64 (Hoffmann et al., 1983) from Drosophila; JNK1 (Sanchez et al., 1994) TGFßR (Wrana et

al., 1992), MAPK (Payne et al., 1991), PKA (Uhler et al., 1986), RAF1 (Bonner et al., 1986), HuCDK2 (Tsai et al., 1991), HuPHK (Hanks, 1989), HuECKH (Lindberg and Hunter, 1990) from humans and CEK8 (Sajjadi and Pasquale, 1993) from chickens. Plants proteins are underlined and CLV1 is in bold lettering.

A



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NVSFTPLFGTISPEIGMLTHLVNL
TLAANNFTGELPLEMKSLTSLKVL
NISNN LTGTFP LKAMVDLEVL
DTYNNNFNGKLPPEMSELKKLKYL
SFGGNFFSGEIPESYGDIQSLEYL
GLNGAGLSGKSPAFLSRLKNLREM
YIGWNSYTGGVPPMFGGLTKLEIL
DMASCTLTGEIPTSLSNLKHLHTL
FLHINNLTGHIPPELSGLVSLKSL
DLSINQLTGEIPQSFINLGNITLI
NLFRNNLYGQIPEAIGELPKLEVF
EVWENNFTLQLPANLGRNGNLIKL
DVSDNHLTGLIPKDLCRGEKLEML
ILSNNFFFGPIPEELGKCKSLTKI
RIVKNLLNGTVPAGLFNLPLVTII
ELTDNFFSGELPVTMSG-DVLDQL
YLSNNWFSGEIPPAIGNFPNLQTL
FLDRNRFRGNIPREIFELKHLSRI
NTSANNITGGIPDSISRCSTLISV
DLSRNRINGEIPKGINNVKNLGTL
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-Φ--N-ΦTG-IP--Φ--Φ--L--L
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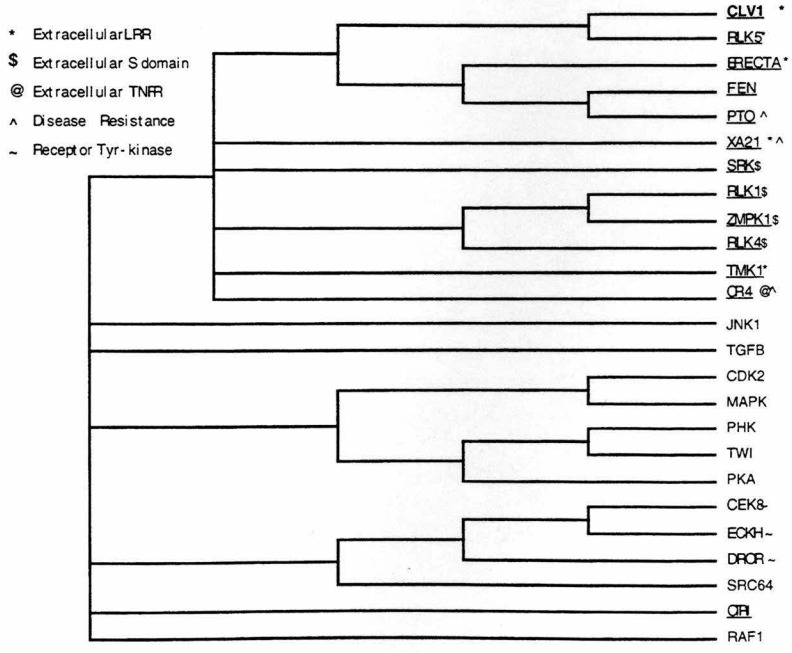

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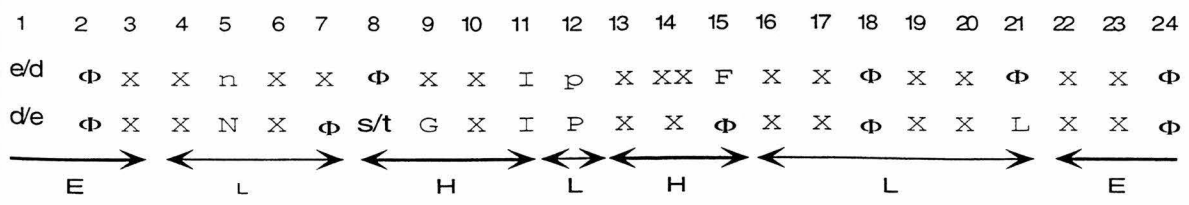

Figure 5. CLV1 mRNA expression patterns in the shoot meristem. A labeled *CLV1*-specific RNA probe was hybridized to serial sections of shoot meristem tissue from wild-type plants. In (A), the line indicates the approximate orientation of serial sections in (B-F). Combined bright-field and dark-field exposures (signal is indicated by red grains) from sections hybridized to antisense (B-F) and sense (G-I) probes. Numbers indicate flower stages (all stages according to Smyth et al., 1990); SM, shoot meristem. Scale Bar represents 40μm.

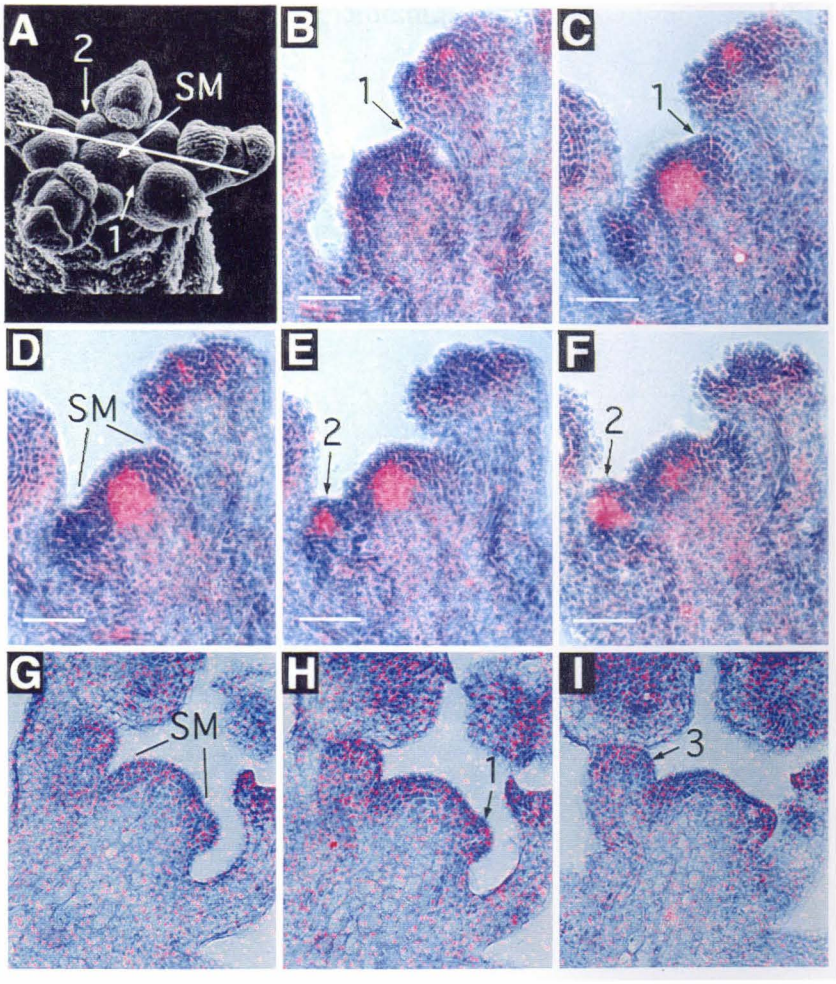
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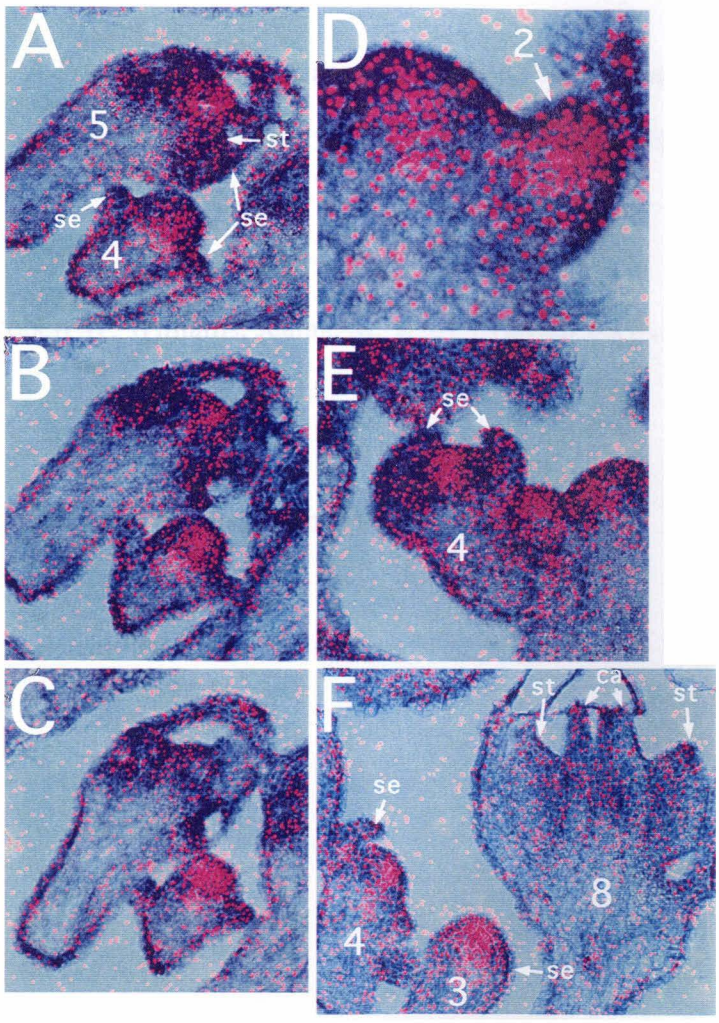
Figure 6. CLV1 mRNA expression patterns in the flower meristem. A labeled *CLV1*-specific antisense RNA probe was hybridized to sections of flower meristem tissue from wild-type plants. (A-C) are serial sections through the same flowers. (D) is a close-up of a stage 2 flower. (E) shows *CLV1* expression in a stage 4 flower and (F) shows a stage 8 flower. All images are shown at the same magnification, except for panel D which is shown at a higher magnification. Numbers indicate flower stages; se, sepal primordium; st, stamen primordium; ca, carpel primordium.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CHAPTER 3

A Possible Role for Kinase-Associated Protein Phosphatase in the *Arabidopsis* CLAVATA1 Signaling Pathway

Robert W. Williams, Jeanne M. Wilson and Elliot M. Meyerowitz

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Abstract

Continuous growth and development in plants are accomplished by meristems, groups of undifferentiated cells that persist as stem cells and initiate organs. While the structures of the apical and floral meristems in dicotyledonous plants have been well described, little is known about the underlying molecular mechanisms controlling cell proliferation and differentiation in these structures. We have previously shown that the *CLAVATA1* (*CLV1*) gene in *Arabidopsis* encodes a receptor kinase-like protein that controls the size of the apical and floral meristems. Here, we show that *KAPP*, a gene encoding a protein phosphatase, is expressed in apical and young floral meristems, along with *CLV1*. Overexpression of *KAPP* mimics the *clv1* mutant phenotype. Furthermore, CLV1 has kinase activity: it phosphorylates both itself and KAPP. Finally, KAPP binds and dephosphorylates CLV1. We present a model where KAPP functions as a negative regulator of the CLAVATA1 signal transduction pathway.

Introduction

Flowering plant development is characterized by the establishment and maintenance of shoot apical meristems (SAMs). These are structures, usually at the growing tips of the plants, that contain slowly dividing stem cells in the center that are surrounded by more mitotically active cells that produce organ primordia (Steeves and Sussex, 1989). In *Arabidopsis*, the SAM is initiated during embryonic growth and then lies dormant until germination. After germination, the SAM initiates leaves, branches and floral meristems in succession. The floral meristems then produce sepal, petal, stamen and carpel primordia. The SAM and floral meristems have a similar structure, but differ in the types of organs initiated, in the phyllotactic pattern of organ initiation, and in ultimate meristem fate. SAMs initiate primordia in a spiral pattern and retain indeterminate growth and meristematic activity. In contrast, floral meristems produce organs in a whorled pattern and are determinate, ceasing growth after the initiation of carpels. Therefore, the control of cell proliferation is developmentally regulated, and in addition, cell division patterns result from communication between cells (Meyerowitz, 1997).

Mutations have been isolated that disrupt meristem structure and/or function in *Arabidopsis*. Among these, mutations in the *CLAVATA1* (*CLV1*) and *CLAVATA3* (*CLV3*) loci result in plants with enlarged apical and floral meristems, indicating a defect in the control of cell proliferation versus differentiation in these meristems (Leyser and Furner, 1992; Clark et al., 1993; Crone and Lord, 1993; Alvarez and Smyth, 1994; Clark et al., 1995). On the basis of double mutant phenotypes and interactions of *clv1* and *clv3* alleles, the *CLV1* and *CLV3* gene products are thought to function in the same pathway (Clark et al., 1995). A third gene, *WUSCHEL* (*WUS*: Laux et al., 1996), has mutant phenotypes opposite those of and epistatic to *clv1*, suggesting that *WUS* may encode a downstream component in the CLV1 pathway.

CLV1 encodes a putative receptor kinase similar in organization and sequence to the *Arabidopsis* RLK5 protein, which was isolated by its sequence similarity to protein kinases but is of unknown function (Clark et al., 1997; Walker, 1993). Both proteins contain an N-terminal signal sequence followed by 21 tandem leucine-rich repeats (LRRs), a membrane-spanning region and a putative cytoplasmic serine/threonine kinase domain. The *CLV1* gene is specifically expressed in the interior layers of the apical and floral meristems but its RNA is not detected in organ primordia (Clark et al., 1997). Therefore, CLV1 may receive a signal that regulates cell proliferation in the meristematic regions.

The RLK5 kinase domain has been shown to autophosphorylate (Horn and Walker, 1994) and interact with a type 2C protein phosphatase, called KAPP (Kinase Associated Protein Phosphatase: Stone et al., 1994). Because CLV1 and RLK5 are closely related members of a larger gene family (Clark et al., 1997), we tested if KAPP may act in the CLV1 pathway. Here we show that the *KAPP* RNA expression domain includes the region of *CLV1* RNA expression and that constitutive *KAPP* expression mimics *clv1* mutant phenotypes. Furthermore, KAPP interacts with the phosphorylated form of the CLV1 kinase domain, and KAPP can dephosphorylate CLV1 *in vitro*. These data suggest that KAPP may function as a negative regulator of the CLV1 signal transduction pathway, as well as in other signaling pathways.

Methods and Materials

In Situ Hybridization

The entire *KAPP* ORF was amplified by polymerase chain reaction (PCR) using KAPP-1 (ATGGCGATGATAGGGATGAAC) and KAPP-6 (TTACAGGGAAGTATCGAAATC) as primers and ligated into a TA cloning vector (Invitrogen). A single plasmid, called pKAPP16, was sequenced to determine the orientation of the insert. Antisense *KAPP* RNA

probe was synthesized using Sp6 RNA polymerase on an *Eco*RV-linearized template. Synthesis of the *CLV1* probe was as described in Clark et al. (Clark et al., 1997). Tissue was fixed, sectioned, hybridized, washed and exposed following the procedure of Drews et al. (1991) with modifications by Sakai et al. (1995).

Construction of Transgenic Plants

The *KAPP* gene was amplified from a cDNA library using the following oligonucleotides with *Bam* HI sites at the ends:

SKAPP1, CGGGATCCAGAGAAGCGAAGACGATGGCG

SKAPP2, CGGGATCCCCTAACATTACAGGGAAGTATC.

The resulting PCR product was digested with *Bam*HI and cloned into pGEM-7zf (Promega). The sequenced insert was transferred, using *Bam*HI, to the Cauliflower Mosaic Virus 35S promoter-containing vector pCGN18 constructed by Leslie Sieburth (Jack et al., 1994). A sense construct was identified, transformed into *Agrobacterium* strain ASE, and transformed into *Arabidopsis thaliana* ecotype Landsberg-*erecta* using the vacuum infiltration method (Bechtold et al., 1993). Transgenic seedlings were selected on Murashige/Skoog (Sigma)+kanamycin plates and transferred to soil. The resulting line was called 35S::*KAPP*.

Construction and Purification of Fusion Proteins

Various regions of *CLV1* and *KAPP* were expressed as glutathione-S-transferase (GST) fusion proteins. The kinase domain of *CLV1* (corresponding to amino acids 657-980; GenBank accession number U96879) was used to make the CKD construct. The kinase interaction domain (KI; amino acids 50-337 of KAPP; see Stone et al., 1994) and the kinase interaction domain plus the protein phosphatase domain (KAPP; amino acids 50-

582) of the *KAPP* cDNA were amplified by PCR using the following oligonucleotides with *Bam* HI sites engineered at each 5' end:

KD1, CGGGATCCGTAGCGATTCGTCAGATGAA

KD4, CGGGATCCAATAGAACGAACCCAGTCTTG

KID1, CGGGATCCGTGGGGGATTTGCAGAGACCA

KID2, CGGGATCCTTAAGCCCCAGGAAGCGGCCAC

SKAPP2, as above.

The PCR products were digested with *Bam* HI and ligated into the *Bam* HI site of pGEX-2TK, which encodes GST, a thrombin cleavage site and a heart muscle kinase (HMK) site upstream and in frame with the *Bam* HI site (Pharmacia Biotech). The *clv1-1* mutation, which results in a Gly to Asp change at position 856 in the CLV1 amino acid sequence (9), was introduced into GST-CKD to make the GST-CKD1-1 construct and the conserved lysine (at position 720 of the CLV1 amino acid sequence) was altered to a glutamic acid to make the GST-CKD_{K720E} construct. All mutant constructs were made using the Chameleon Double Stranded Site Directed Mutagenesis kit following the manufacturer's instructions (Stratagene). All plasmids were verified by sequencing, and transformed into *E.coli* strain ADA 94 (Novagen) for protein expression.

GST-fusion proteins were isolated using a modified protocol from Chang et al. (1992). *E. coli* cells were grown in 200 ml of Luria-Bertani medium to approximately OD_{600} 0.8 and then induced with 0.1 mM isopropyl-ß-D-thiogalactopyranoside for 2-3 hours at 37° C. The induced cells were harvested by centrifugation, resuspended in 10ml of lysis buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 0.2% Tween 20 with 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.50 mg/ml Pefabloc SC (Boehringer Mannheim) and 10 µg/ml pepstatin as protease inhibitors, lysed by sonication and separated into soluble and insoluble fractions by centrifugation. The soluble GST-fusion proteins were purified from the supernatant using Glutathione Sepharose 4B (Pharmacia Biotech) following the manufacturer's instructions.

CLV1 Autophosphorylation and Interactions with KAPP

The GST-CKD, GST-CKD1-1 and GST-CKD_{K720E} fusion proteins were assayed for autophosphorylation by incubation in 30 µl of 20 mM Tris (pH7.5), 100 mM NaCl, 12 mM $MgCl_2$ with 10 µCi of [γ-^{32}P]ATP for 1 hour at room temperature. Samples were boiled in SDS loading buffer and analyzed by SDS-PAGE. Coomassie stained gels were dried and exposed to film. CLV1 transphosphorylation was assessed by mixing CKD_{K720E} protein, removed from GST by thrombin cleavage, with GST-CKD in an autophosphorylation reaction. CKD_{K720E}-dependent inhibition of GST-CKD autophosphorylation was demonstrated by removing the CKD_{K720E} protein from GST by thrombin cleavage, then adding increasing amounts of CKD_{K720E} to GST-CKD during autophosphorylation reactions. Phosphoamino acid analysis was performed as described by Boyle et al. (1991).

GST-KI was phosphorylated with heart muscle kinase (Sigma) in 20 mM Tris (pH7.5), 100 mM NaCl, 12 mM $MgCl_2$ with 10 µCi of [γ-^{32}P]ATP, and then eluted from the matrix by washing with 20 mM Tris (pH7.5), 100 mM NaCl, 12 mM $MgCl_2$, 20 mM glutathione. The resulting supernatant was used to probe various GST fusion proteins bound to nitrocellulose filters as described by Stone et al. (1994). Phosphorylation dependence of the CLV1/KAPP interactions was tested by dephosphorylating immobilized GST-CKD with 20U of alkaline phosphatase with and without 100mM PO_4^- as an inhibitor of alkaline phosphatase. GST-CKD was washed after phosphatase treatment and incubated with cold ATP in a standard autophosphorylation reaction. CLV1-dependent KAPP phosphorylation was assessed by adding GST-KAPP to GST-CKD, GST-CKD1-1 and GST-CKD_{K720E} during a standard autophosphorylation reaction.

GST-KAPP dependent dephosphorylation of GST-CKD was tested using a modified protocol from Stone et al. (1994). The GST-CKD protein, still bound to the

Glutathione Sepharose 4B matrix, was allowed to autophosphorylate in the presence of [γ-^{32}P]ATP, then washed three times in 20 mM Tris (pH7.5), 0.1% ß-ME. Labeled GST-CKD, still immobilized on the matrix, was incubated with and without GST-KAPP in 20 mM Tris (pH7.5), 0.1% ß-ME with either 10 mM EDTA or 10 mM $MgCl_2$/10 mM $MnCl_2$ for 2 hours at room temperature.

Results

KAPP Expression Patterns

We determined the pattern of *KAPP* mRNA expression by in situ hybridization. Since *KAPP* does not appear to cross-hybridize to any other *Arabidopsis* DNA sequences at low stringency (RWW and EMM, data not shown), the entire *KAPP* ORF was used to make an antisense RNA probe that was hybridized to 8 µm sections of wild-type and *clv1-4* inflorescence tissue. In wild-type tissue, *KAPP* was expressed throughout the SAM and stage 1-2 flowers (Smyth et al., 1990) but appeared to be excluded from the sepal primordia of stage 3 flowers (Figure 1*A-D*). *CLV1* is expressed in a much more restricted domain, the central cells of the SAM and young floral meristems (Figure 1*E*: Clark et al., 1997). Therefore, the *CLV1* expression domain is encompassed within that of *KAPP*. Below the SAM, *KAPP* RNA was detected in the provascular tissue, but not differentiated vascular or pith tissue (Figure 1*A-C, F*). After stage 5 of flower development, *KAPP* RNA levels appeared to decrease with only weak signal detected in the stamen and carpel primordia (Figure 1*G*). Later in flower development, *KAPP* was expressed in the developing pollen sacs and ovules (Figure 1*H*). *KAPP* expression was maintained during ovule development (Figure 1*I*).

KAPP has a similar expression pattern in *clv1-4* tissue as it does in wild-type tissue. It is expressed throughout the enlarged apical and floral meristems (Figure 1*J*) as

well as in the pollen sacs, ovules and central proliferating dome of the mature flowers (Figure 1*K*). This indicates that *clv1* mutations do not dramatically affect the level or the spatial regulation of *KAPP* expression. In situ hybridization of *KAPP* to *clv3-2* tissue results in an RNA expression pattern similar to that in *clv1-4* tissue (data not shown). All expression patterns are specific for *KAPP* and not due to background, as a *KAPP* sense probe gave very little signal (Figure 1*L*).

KAPP Transgenic Studies

To identify possible roles of *KAPP* in meristem and flower development, we constructed transgenic lines that express the full-length *KAPP* cDNA under control of the strong, constitutive 35S CaMV promoter (Odell et al., 1985). Two independent lines exhibited a dominant, heritable floral phenotype that co-segregated with the T-DNA. Both lines appear similar, so only one line was analyzed in detail.

The 35S::*KAPP* line has a floral phenotype reminiscent of that caused by weak loss-of-function *clv1* alleles (Clark et al., 1993). Flowers of the transgenic plant have a small but consistent increase in the number of carpels compared to wild type (Figure 2*A-C*). Wild type Landsberg-*erecta* plants have 2.0+/-0.0 carpels per flower. On average, 35S::*KAPP* expressing plants have 0.30+/-0.105 extra carpels per flower, and plants homozygous for the weak *clv1-7* allele have 0.75 +/-0.143 extra carpels relative to wild type. The only other observed phenotypic alteration in the 35S::*KAPP* line was the presence of occasional petalloid stamens in the third whorl of the flowers (data not shown). Flowering time, apical dominance, and male and female fertility appear normal.

CLV1 Autophosphorylation

To test for CLV1 protein kinase activity, we expressed three different versions of the CLV1 kinase domain in *E. coli.* The kinase domain from CLV1 was used to make a GST fusion protein, called GST-CKD (CLV1 Kinase Domain). Fusion protein was purified from *E. coli* and shown to autophosphorylate on serine residues (Figure 3*A-B*). This CLV1 kinase activity requires Mn^{2+} or Mg^{2+} (data not shown). The *clv1-1* mutation results in a protein with an Asp substituted for the first Gly in box VIII of the kinase domain (Clark et al., 1997; Hardie and Hanks, 1995). We made the CLV1-1 form of the kinase domain fused to GST, GST-CKD1-1, and found that it is able to autophosphorylate but at greatly reduced levels (Figure 3*A*). When the catalytic Lys residue was changed to glutamic acid (GST-CKD_{K720E}), the resulting protein was unable to autophosphorylate (Figure 3*A*).

Genetic evidence suggests that the CLV1 protein may function as an oligomer (4, 7). The closely related RLK5 kinase domain can undergo intermolecular phosphorylation (Horn and Walker, 1994), and the rate at which GST-CKD autophosphorylates appears to be dependent on the concentration of the fusion protein (data not shown), suggesting a second order reaction. We therefore tested the ability of CLV1 to transphosphorylate. The inactive CKD_{K720E} protein, when removed from the GST-moiety by thrombin cleavage, could be phosphorylated in the presence of the active GST-CKD protein (Figure 3*C*). Furthermore, GST-CKD autophosphorylation is reduced when incubated with increasing amounts of CKD_{K720E} (Figure 3*D*). This indicates that CLV1, like RLK5 (Horn and Walker, 1994), can interact intermolecularly and undergo transphosphorylation.

CLV1/KAPP Interactions

The region of the KAPP protein defined to be the Kinase Interaction Domain (Stone et al., 1994) was also used to make a GST-fusion protein, called GST-KI. The KI protein could be labeled at an engineered HMK site. The resulting GST-KI protein interacted with GST-CKD (Figure 4*A*) in a filter binding assay. GST-KI did not bind to either GST-CKD1-1 or

GST-CKD$_{K720E}$ at detectable levels (Figure 4*A*), suggesting that the introduced mutations alter residues or structures that are required for the interaction, or that the CLV1/KAPP interaction is dependent on phosphorylation of the CLV1 kinase domain. To see if this interaction was dependent on the phosphorylation state of the CLV1 protein, we treated GST-CKD with alkaline phosphatase (AP) alone or with AP plus PO_4^- (PO_4^- is an inhibitor of the phosphatase). GST-KI had a reduced interaction with the dephosphorylated GST-CKD as compared to phosphorylated GST-CKD (Figure 4*A*). This interaction could be recovered by allowing the dephosphorylated GST-CKD protein to autophosphorylate (Figure 4*A*).

The ability of the CLV1 kinase domain to phosphorylate KAPP was also tested. The GST-CKD protein was able to phosphorylate the full length KAPP protein fused to GST (Figure 4*B*). Consistent with their abilities to autophosphorylate, GST-CKD1-1 could phosphorylate GST-KAPP at reduced levels and the GST-CKD$_{K720E}$ protein could not phosphorylate GST-KAPP at any detectable level (Figure 4*B*). GST-CKD protein does not phosphorylate GST alone (data not shown).

The KAPP protein had previously been shown to have protein phosphatase activity (Stone et al., 1994). To further investigate the interaction between the CLV1 kinase domain and KAPP, we tested KAPP's ability to dephosphorylate GST-CKD *in vitro*. The phosphorylated GST-CKD protein was incubated with the GST-KAPP fusion protein in the presence and absence of divalent cations. The CKD protein is dephosphorylated by KAPP, and like other type 2C protein phosphatases, KAPP activity is dependent on Mg^{2+} and/or Mn^{2+} (Figure 4*C*).

Discussion

We have started to investigate the molecular mechanisms of CLV1 signal transduction. We have shown that the CLV1 kinase domain can undergo intermolecular autophosphorylation,

which suggests that the active CLV1 protein may function as a multimer. The KAPP protein binds the phosphorylated form of CLV1 and exhibits phosphatase activity on the CLV1 protein. *CLV1* is expressed in regions that also express *KAPP*, although the *KAPP* expression domain is much larger. Finally, transgenic plants overexpressing *KAPP* mimic the floral defects seen in weak *clv1* mutant plants. These data taken together argue that KAPP may function as a negative regulator of the CLV1 signal transduction pathway. A model for the CLV1 signal transduction pathway is given in Figure 5. Further experimentation will be needed to test the *in vivo* validity of this model, as the data are suggestive but not conclusive. In particular, KAPP may function in a process parallel to CLV1.

Mechanisms of CLV1 Action

The CLV1 biochemical activity may account for the previously observed phenotype seen in the *clv1-1* mutant. The *clv1-1* allele is predicted to encode a full length protein with a Gly856 to Asp missense mutation in a conserved residue of subdomain VIII (Clark et al., 1997), which is thought to be involved in the recognition of protein substrates (Hardie and Hanks, 1995). *clv1-1* homozygous plants have an intermediate phenotype indicating that it is not a null allele. Also, *clv1-1*/+ heterozygous plants have a weak mutant phenotype (Clark et al., 1993; Clark et al., 1995), whereas the phenotypically stronger *clv1-4* allele does not have a heterozygous mutant phenotype, indicating that the CLV1-1 protein may act in a dominant negative manner. This suggests that CLV1 may function as multimer.

The CLV1-1 form of the kinase domain can autophosphorylate, but only at low levels, consistent with *clv1-1* not being a complete loss-of-function allele. We have also shown that the CLV1 kinase domain can transphosphorylate an inactive form of the protein. Furthermore, large amounts of the inactive (CKD_{K720E}) kinase can inhibit the autophosphorylation of the wild-type protein. These data, consistent with the genetic data,

argue that the CLV1 protein may function as a multimer. Like receptor-tyrosine kinases in animals (Carpenter, 1987), the CLV1 extracellular domain may inhibit dimerization, which is overcome by ligand binding. Upon oligomerization, the cytoplasmic domains may autophosphorylate and then recruit specific downstream components.

We have also shown that CLV1 is capable of phosphorylating KAPP, but it remains to be seen if CLV1 phosphorylates other substrates. As will be discussed below, KAPP is likely to be a negative regulator of the CLV1 pathway. No downstream components of the pathway have been identified, but epistasis analysis suggests the product of the *WUS* (Laux et al., 1996) gene as a candidate (Figure 5).

Mechanisms of KAPP Action

Signaling pathways, like many developmental mechanisms, need both positively acting components and negative regulators. Positively acting components are needed to transduce a signal to the nucleus for changes in gene expression or to the cytoskeleton for changes in cell shape. Negative regulators are needed to attenuate the signaling cascade and to repress the pathway when the signal is no longer present. The KAPP protein may function as a negative regulator of the CLV1 signal transduction pathway. The region of *KAPP* expression includes the *CLV1* expression domain, showing that the two genes are at least expressed in the same cells. Moreover, high levels of *KAPP* gene expression can mimic *clv1* mutants. Mutations in the *CLV1* or *CLV3* genes do not appear to affect the level of *KAPP* expression. So the similarity of *clv1* and 35S::*KAPP* phenotypes is probably not the indirect result of increased *KAPP* expression in *clv1* or *clv3* plants.

More importantly, the KAPP protein binds to the phosphorylated form of the CLV1 kinase domain. The KAPP protein does not appear to merely bind phosphoserine and/or phosphothreonine, since KAPP does not interact with all tested kinases (Braun et al., 1997), so there must be other sequence requirements for KAPP function. However, KAPP

can bind to denatured proteins and thus is not dependent on a proper structural conformation (Figure 4*A*: see also Stone et al., 1994), suggesting that KAPP may recognize a short amino acid sequence. Since the KAPP protein exhibits phosphatase activity towards the CLV1 kinase domain, KAPP may function *in vivo* by directly dephosphorylating CLV1, thus preventing other downstream components from receiving the CLV1 signal. KAPP may dephosphorylate other components of the pathway as well. The KAPP protein is phosphorylated in the presence of CLV1, suggesting that the activity of KAPP may be modulated in response to the activated CLV1 receptor. However, our data suggest that phosphorylation of KAPP is not required for KAPP to dephosphorylate CLV1.

Other Roles for KAPP

There are several lines of evidence indicating that KAPP may function in a number of different signaling pathways. First, KAPP interacts with RLK5, CLV1 and several other receptor-kinases (Braun et al., 1997). RLK5 and CLV1 are members of a large family of LRR containing receptor kinases, of which there are members in *Arabidopsis* that are closely related to CLV1, and others closely related to RLK5 (Clark et al., 1997: RWW and EMM, unpublished data). So far, no role has been ascribed to RLK5. Second, based on low stringency hybridization experiments and EST database searches, there does not appear to be another *KAPP*-like gene in the *Arabidopsis* genome (RWW and EMM, unpublished data). It is therefore possible that KAPP is a promiscuous molecule that regulates a number of signaling pathways.

The expression data for *KAPP* supports such an idea. Previous work has shown that the *KAPP* mRNA is found in root and rosette tissue (Stone et al., 1994). In situ hybridization to inflorescence tissue shows that *KAPP* is expressed throughout the SAM and young floral primordia and in a punctate ring down the stem. *CLV1* is expressed in a

much smaller region of the SAM and floral meristems. Later in flower development, *KAPP* is expressed in the pollen sacs and in the placenta and ovules of the carpel, thus suggesting a role for KAPP in the formation of gametes.

In the 35S::*KAPP* lines, we did not observe a defect in female or male fertility. This may be due to higher endogenous levels of *KAPP* mRNA in these cells and/or a very sensitive requirement for lower levels of *KAPP* mRNA in the *CLV1*-expressing cells. Alternatively, the 35S promoter may not be active in these cells. There were also minor floral organ identity defects in the 35S::*KAPP* line, suggesting that KAPP can function in *CLV1* non-expressing cells. The absence of observable defects outside the flower indicates that *KAPP* overexpression does not affect all cell types and likely needs other factors to function. It is interesting to speculate that the relatively large and expanding family of LRR-kinases in plants may play a variety of roles in communicating information needed for proper cell proliferation, elongation and differentiation, and it is possible that KAPP may be an important negative regulator of a number of these kinases.

Acknowledgments

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Figure 1. Expression of *KAPP* in wild type and *clv1-4* tissue. Labeled *KAPP* and *CLV1*-specific RNA probes were used for hybridization to sections of inflorescence meristem and floral tissue. (*A-C*) Serial sections of wild type tissue showing *KAPP* RNA is detected throughout the apical and young floral meristems and down the provascular tissue. (*D*) Section of a wild-type stage 3 flower where KAPP expression is reduced in sepal primordia. (*E*) *CLV1* mRNA expression in the center of the apical and young floral meristems only. *CLV1* is not expressed in stage 1 flowers. (*F*) Transverse section of the stem, below the SAM, showing the punctate ring of *KAPP* expression in the provascular tissue. (*G*) shows *KAPP* weak expression in the stamen and carpel primordia of a stage 7 flower; (*H*) shows *KAPP* expression in the pollen sacs and carpels of a stage 9 flower; (*I*) shows *KAPP* expression in the developing ovules. (*J*) *KAPP* is expressed throughout the enlarged SAM of a *clv1-4* mutant. (*K*) *KAPP* is expressed in the central meristematic regions in mature *clv1-4* flowers. (*L*) A *KAPP* sense probe hybridized to a wild-type inflorescence section. All photos are combined bright-field and dark-field exposures, with signal indicated by yellow grains. PV, provascular; SAM, shoot apical meristem; SP, sepal primordia; St, stamen, Ca, carpel; PS, pollen sac; Pl, placenta; Ov, ovule; Si, silique wall; CM, central meristematic regions. Numbers indicate stages of floral development (all stages according to Smyth et al., 1990). Scale bars represent 40μm.

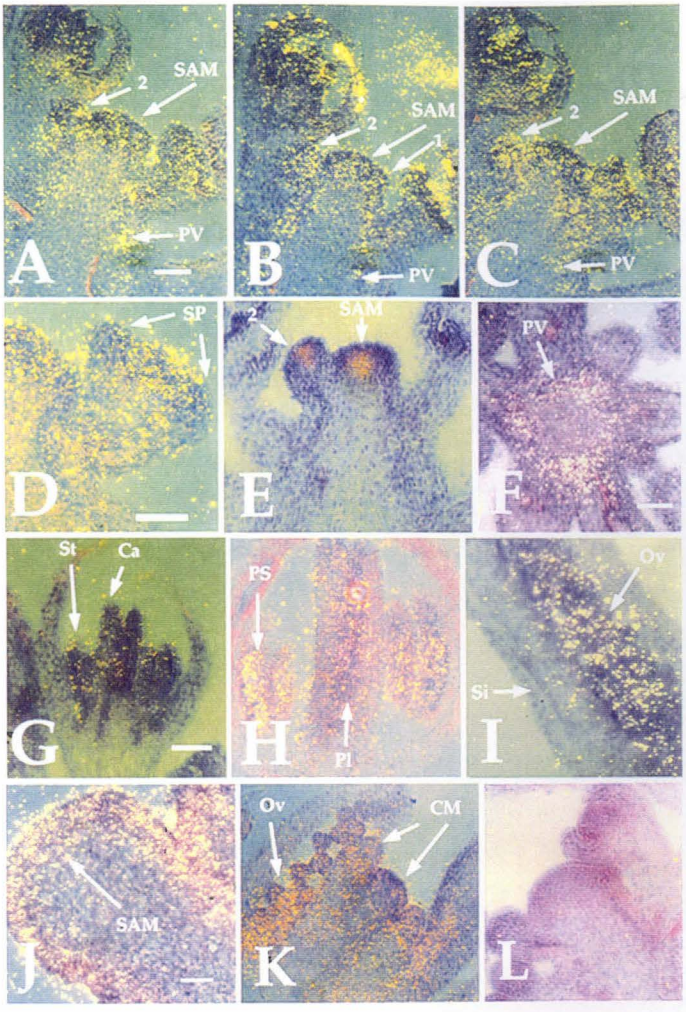

A
SAM
2
PV
B
SAM
2
1
PV
C
SAM
2
PV
D
SP
E
SAM
2
F
PV
G
St
Ca
H
PS
Pl
I
Ov
Si
J
SAM
K
Ov
CM
L

Figure 2. 35S::*KAPP* phenotype. A comparison of (*A*) wild-type, (*B*) 35S::*KAPP* and (*C*) *clv1-1* siliques. (*A*) A typical silique from a wild-type Landsberg-*erecta* flower is composed of two fused carpels. The 35S::*KAPP* line has on average 2.3 carpels per flower; panel (*B*) shows a silique comprised of three fused carpels. (*C*) *clv1* mutants have extra carpels that fuse into a functional silique. This representative *clv1-1* flower contains four carpels.

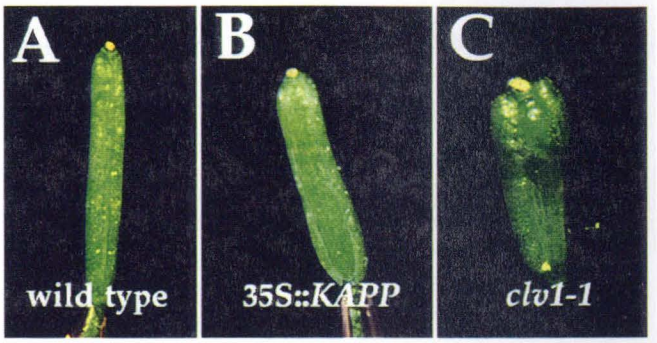

A
wild type
B
35S::KAPP
C
clv1-1

Figure 3. Autophosphorylation of the CLV1 kinase domain. (*A*) GST fusion-proteins containing the wild type (lane 1), CLV1-1 (lane 2) and CKD_{K720E} (lane 3) forms of the CLV1 kinase domain were allowed to phosphorylate in the presence of [γ-^{32}P]ATP. The upper panel is the Coomassie Blue-stained gel showing the relative amounts of fusion protein in each lane. The lower panel shows the autoradiograph. (*B*) The wild-type CLV1 kinase domain was allowed to autophosphorylate, then hydrolyzed. The products of the hydrolysis reaction were analyzed by electrophoresis on a TLC plate. Only serine residues were phosphorylated. (*C*) Autophosphorylation reactions containing CKD_{K720E} alone (lane 1), CKD_{K720E} plus GST-CKD (lane 2) and GST-CKD alone. (*D*) Increasing amounts of CKD_{K720E} protein were added to a constant amount of GST-CKD during autophosphorylation reactions. The approximate molar ratios of CKD_{K720E}:GST-CKD are 0:1 (lane 1), 1:1 (lane 2), 2:1 (lane 3) and 4:1 (lane 4). The arrow shows the position of the full length GST-CKD protein.

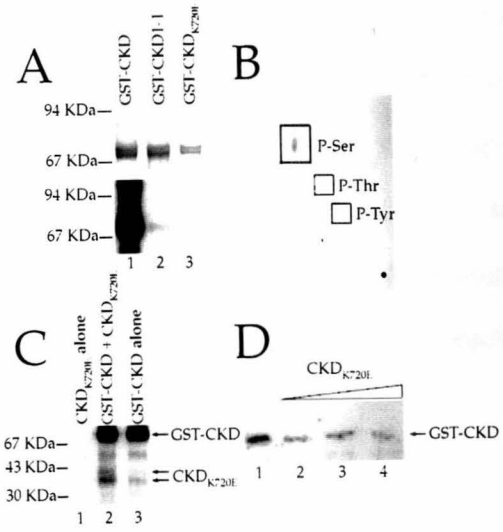

A
GST-CKD
GST-CKD1-1
GST-CKD$_{K720E}$
94 KDa
67 KDa
94 KDa
67 KDa
1 2 3
B
P-Ser
P-Thr
P-Tyr
C
CKD$_{K720E}$ alone
GST-CKD + CKD$_{K720E}$
GST-CKD alone
67 KDa
43 KDa
30 KDa
GST-CKD
CKD$_{K720E}$
1 2 3
D
CKD$_{K720E}$
GST-CKD
1 2 3 4

Figure 4. KAPP interactions with the CLV1 kinase domain. (*A*) GST-KI protein was labeled at the HMK site with [γ-^{32}P]ATP and incubated with a filter containing immobilized GST-CKD (lane 1), GST-CKD1-1 (lane 2), GST-CKD_{K720E} (lane 3), GST-CKD treated with alkaline phosphatase (lane 4), GST-CKD treated with alkaline phosphatase in the presence of 100mM PO_4^- (lane 5) and GST-CKD treated with alkaline phosphatase, then allowed to autophosphorylate in the presence of $MnCl_2$ and ATP (lane 6). The upper panel shows the Coomassie Blue-stained gel showing the relative amounts of fusion protein in each lane. The lower panel shows the autoradiograph. (*B*) The GST-KAPP protein was incubated alone (lane 1) or with GST-CKD (lane 2), GST-CKD1-1 (lane 3), or GST-CKD_{K720E} (lane 4) during an autophosphorylation reaction. Equal amounts of GST-CKD, GST-CKD1-1 and GST-CKD_{K720E} were added in each reaction. (*C*) 32P-labeled GST-CKD was incubated with full-length GST-KAPP protein (lanes 1 and 2), or without GST-KAPP (lanes 3 and 4) and with either $MgCl_2$/$MnCl_2$ (lanes 1 and 3) or EDTA (lanes 2 and 4). Lane 5 is an untreated control. The upper panel is the Coomassie Blue-stained gel showing the approximately equal loading of GST-CKD in each lane. The lower panel is the autoradiogram.

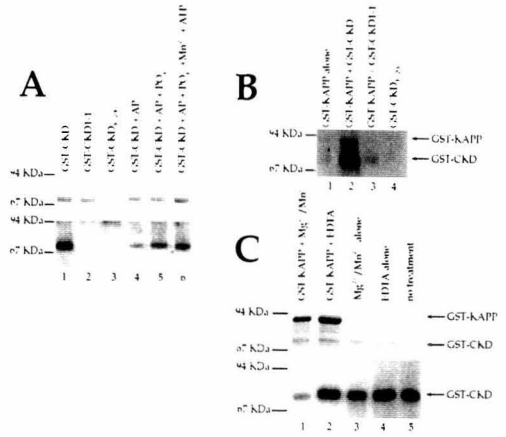
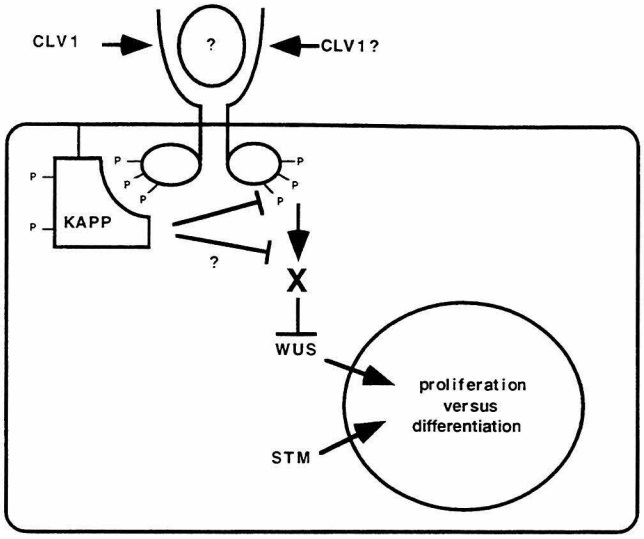

A
B
C
94 KDa
67 KDa
GST-KAPP
GST-CKD
1
2
3
4
5
6

Figure 5. Model of CLV1 and KAPP Action. The model predicts that CLV1, upon binding a peptide ligand, dimerizes and allows intermolecular phosphorylation of the cytoplasmic kinase domains. The activated receptor then binds and phosphorylates KAPP. The KAPP protein may then modify the CLV1 signal by directly dephosphorylating CLV1. The CLV1 kinase domain probably interacts with other positively acting components (X) which may or may not be dephosphorylated by KAPP. Based on epistasis analysis, WUS may function downstream of CLV1 to promote cell divisions. SHOOTMERISTEMLESS (STM) may function independently but competitively with the CLV1 signaling pathway to promote cell division (Clark et al., 1996).


CLV1
?
CLV1?
P
P
P
P
P
P
P
P
KAPP
?
X
WUS
proliferation
versus
differentiation
STM

CHAPTER 4

CLAVATA3 Acts at or Upstream of *CLAVATA1*

Robert W. Williams and Elliot M. Meyerowitz

Abstract

Genetic analysis in *Arabidopsis* has uncovered several genes required to regulate the balance between stem cell proliferation and organogenesis in the shoot apical and floral meristems. Mutations in the *CLAVATA1* (*CLV1*) or *CLAVATA3* (*CLV3*) genes result in plants with overgrown meristems. The *CLV1* gene encodes a putative receptor kinase. Double mutant analysis suggests that *CLV1* and *CLV3* may act in a common pathway but the order in which the gene products function relative to each other is not known. We show here that wild-type *CLV3* is not required for *CLV1* transcription and translation. Furthermore, we show that cosuppression of a protein phosphatase thought to be a negative regulator of CLV1 suppresses both *clv1* and *clv3* alleles. Therefore, *CLV3* likely functions upstream of *CLV1* and is a candidate to encode a CLV1 ligand.

During plant embryogenesis, two pools of stem cells are set aside that ultimately produce the adult body plan. The shoot apical meristem (SAM) contains a central region of slowly dividing cells surrounded by a region where rates of mitosis increase and organs are initiated (Steeves and Sussex, 1989). The rate at which cells divide is tightly coupled to the rate of cell differentiation such that the SAM meristem remains roughly the same size throughout the life span of the plant. The molecular mechanisms used to regulate meristem growth and maintenance are only beginning to be understood and how information regarding proliferation and differentiation in the SAM is communicated between cells is not known.

Among the many known genes required for proper meristem patterning and growth in *Arabidopsis*, mutational analysis has shown that the *CLAVATA* (*CLV*) loci are required to maintain the size of the meristem. Mutations in the *CLV1* or *CLV3* genes result in plants that have enlarged SAMs and floral meristems that lead to stem fasciation and the production of supernumerary floral organs (Clark et al., 1993; Clark et al., 1995). Genetic interactions between *clv1* and *clv3* alleles suggests that the wild-type gene products may function in the same pathway. *CLV1* encodes a putative receptor-kinase (Clark et al., 1997) which immediately suggests that *CLV3* may encode either a ligand or a downstream component in the CLV1 signaling pathway. However, there are no gain-of-function alleles at either loci so the genes cannot be ordered in a genetic pathway.

To rule out the possibility that *CLV3* is required for *CLV1* expression, and thus the similar mutant phenotypes are a result of reduced *CLV1* expression in a *clv3* plants, we analyzed the pattern of *CLV1* RNA expression by *in situ* hybridization and the pattern of CLV1 protein expression by immuno-histochemistry in *clv3* mutant tissue. The *CLV1* gene is expressed in the subepidermal cells in the central region of wild-type SAMs and young floral meristems (Figure 1A; Clark et al., 1997). Similarly, the staining of wild-type tissue with CLV1 specific antibodies shows that the protein is expressed in the SAM and floral meristems (Figure 1B). It is interesting to note that the protein appears to be detected in

cells in which the RNA is not detected; specifically in the epidermal cell layers and in cells further down the flanks of the SAM and young floral meristems. In *clv3-2* tissue, *CLV1* is expressed at comparable levels as in wild-type. The SAMs and young floral buds are enlarged in *clv3* plants and *CLV1* is expressed throughout the enlarged meristems (Figure 1C-D). In a formal sense, *CLV3* is required to restrict the domain of CLV1 expression but it is clearly not required for *CLV1* expression. Similarly, *CLV1* expression, which is normally terminated in wild-type flowers at the time of carpel initiation, is maintained in the central meristematic dome in the indeterminate *clv3* flowers (Figure 1E-F). So *CLV3* is required to properly down-regulate *CLV1* expression during flower development. The expression of *CLV1* in *clv1-4* tissue looks very similar to that seen in *clv3-2* suggesting that any reduction in the CLV1/CLV3 signaling pathway result in the enlargement of *CLV1* expressing cells.

The CLV1 receptor is thought to oligomerize and autophosphorylate in response to a ligand. This is based on the fact that the CLV1 kinase domain autophosphorylates in vitro (Williams et al., 1997) and that a mutation that reduces kinase activity has a weak dominant negative phenotype (Clark et al., 1995). Presumably the activated receptor then recruits downstream components that transduce the extracellular signal. The kinase-associated protein phosphatase (Stone et al., 1994; KAPP) is thought to interact with the CLV1 kinase domain and function to negatively regulate the pathway (Williams et al., 1997).

Arabidopsis lines expressing the full length *KAPP* gene driven by the 35S promoter have an extra carpel phenotype, very similar to weak *clv1* alleles (Williams et al., 1997). This phenotype appears to be dose-dependent because the average number of carpels per flower increased when the transgene was homozygosed. Specifically, plants carrying no copies of the transgene have 2.0 carpels/flower; plants with one copy of the transgene have 2.3 carpels/flower and plants harboring two copies of the transgene have 3.3 carpels/flower (Table 1). To further test the hypothesis that KAPP functions in the CLV1/CLV3 pathway, we analyzed plants that are homozygous for the 35S::*KAPP* transgene and homozygous for

clv1 or *clv3* alleles. The addition of the 35S::*KAPP* construct did not significantly increase the number of carpels per flower in the strong *clv1-4* or *clv3-2* alleles, and only increased the number of carpels in the slightly weaker *clv3-1* allele to approximately the number seen in the strong *clv3-2* allele (Table 1). This suggests that *KAPP* can only increase the number of carpels/flower when there is some *CLV1* and/or *CLV3* activity, so all three proteins likely function in a common pathway.

We then generated 40 transgenic lines that expressed a truncated *KAPP* cDNA under control of the 35S promoter (35S::*KI*) in a *clv1-4* background. Approximately 8 of those lines had a partially suppressed *clv* phenotype (Figure 2). One line was analyzed further. Northern blot analysis showed that the level of *KAPP* RNA was reduced suggesting that in these lines, the transgene was cosuppressing the endogenous *KAPP* gene (data not shown). The average number of carpels per flower was reduced to 2.9 (Table 1). This is consistent with *KAPP* functioning as a negative regulator of the *CLV1* signaling pathway. This line also had defects in plant height and fertility, consistent with *KAPP*s' potential roles in *CLV1* independent pathways (Braun et al., 1997; Williams et al., 1997).

The transgene was then introduced to plants *clv3-1* plants. Of 36 lines, 5 showed a partially suppressed *clv1* phenotype (Table 1). It is assumed that these lines are also cosuppressed but Northern blot analysis has not yet been done. Since reduction of *KAPP* expression suppresses mutations in both the *CLV1* and *CLV3* genes, *CLV3* likely functions at or upstream of *CLV1*.

All these data suggests that CLV3 is not so far upstream as to affect the expression of *CLV1* nor does it function downstream of KAPP which is thought to directly interact with the cytoplasmic domain of CLV1. It will be interesting to see if ligand independent activation of CLV1 can suppress *clv3* alleles.

Methods and Materials

In situ hybridization was done according to Drews et al. (1991) with modifications by Sakai et al. (1995).

CLV1 antisera was raised by injecting rabbits with 6-His tagged fusion proteins. Separately polyclonal antibodies were generated to the leucine-rich repeat portion of the CLV1 protein and the kinase domain of CLV1 following the frameshift in *clv1-6*. Rabbits were injected with approximately 100μg of purified fusion protein every 4 weeks. In between the injections, the rabbits were bled. Immunolocalizations were done on 8μm paraplast sections with a 1:2000 dilution of antisera and detected using the Vectastain kit following the manufacturers' instructions.

The truncated *KAPP* transgene was constructed by PCR amplifying the membrane anchor and KI domain of KAPP without the protein phosphatase encoding domain, using the high fidelity Pfu enzyme, and then ligating the *Bam*HI digested product into the *Bam*HI site in the 35S promoter/3' nos containing vector pCGN18. The orientation of the insert was checked and the resulting plasmid, with the truncated *KAPP* cDNA in the sense orientation, was transferred to *Agrobacterium tumefaciens* ASE. Transgenic plants were obtained by vacuum mediated *in planta* transformation following the procedure of Bechtold et al. (1993) and subsequent selection of seeds on kanamycin.

Acknowledgments

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Williams, R. W., Wilson, J. M. and Meyerowitz, E. M. (1997). A possible role for kinase-associated protein phosphatase in the *Arabidopsis* CLAVATA1 signaling pathway. Proc. Natl. Acad. Sci. **94**, 10467-10472.

Table 1. Affects of *KAPP* transgenes on the clv mutant phenotype. The average number of carpels (+/- standard deviation) per flower.

genotype	no transgene	35S::*KAPP*	35S::*KI*
+/+	2.0+/-0.0	3.3+/-1.0	n.d.
clv1-4	5.4+/-0.7	5.7+/-0.9	2.9+/-0.8
clv3-1	5.2+/-0.8	5.9+/-0.8	3.5+/-1.0
clv3-2	6.1+/-0.5	6.2+/-0.7	n.d.

Figure 1. CLV1 expression in *clv3-2* plants. RNA in situ hybridizations with a *CLV1* specific probe (A, C, E) and immunolocalizations with a polyclonal antibody made against a region of the CLV1 kinase domain (B, D, F). (A, B) wild-type inflorescence meristem and young floral meristems. (C, D) *clv3-2* inflorescence meristems. (E, F) stage 9 *clv3-2* flowers. IM, inflorescence meristem; CM, central meristematic dome; numbers refers to stages of floral development.

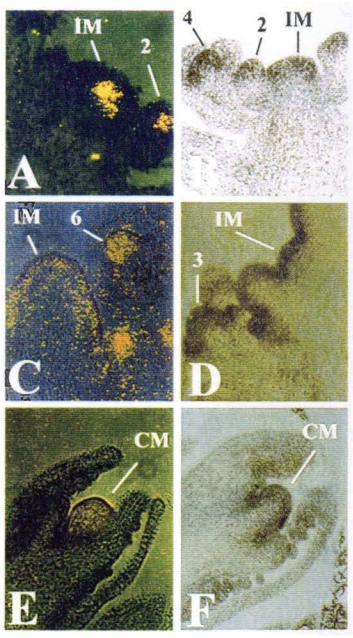
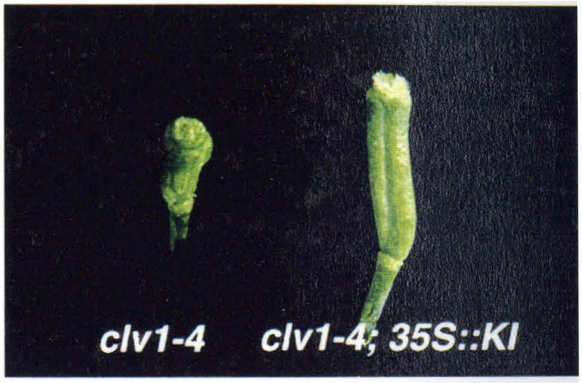

IM
2
A
4
2
IM
B
IM
6
C
IM
3
D
CM
E
CM
F

Figure 2. Partial suppression of the *clv1-4* phenotype. Left, a typical gynoecium from a *clv1-4* plant showing 5 fused carpels. Right, a gynoecium from a *clv1-4* plant also carrying a truncated *KAPP* transgene.


clv1-4
clv1-4; 35S::KI

CHAPTER 5

Members of the Leucine-Rich Repeat Receptor Kinase Gene Family Play Diverse Roles During Arabidopsis Development

Robert W. Williams and Elliot M. Meyerowitz

Abstract

The *CLAVATA1*, *ERECTA* and *BRI1* genes from *Arabidopsis* have been shown to encode receptor kinase-like proteins required for proper plant development. Furthermore, other leucine-rich repeat kinases of unknown function have been isolated from *Arabidopsis*. Here we describe the isolation of two additional genes from *Arabidopsis* that encode leucine-rich repeat kinases. Sequence comparisons suggest that these genes and the previously identified leucine-rich repeat kinases belong to several subfamilies. Expression patterns show that this family of genes has assumed diverse roles in plant development.

Introduction

Plasma membrane associated receptor-kinases have long been known to sense extracellular signals and initiate intracellular signaling cascades to regulate key aspects of cell proliferation and differentiation during animal development. For example, receptor tyrosine kinases (RTK) are known to dimerize in response to specific ligands, such as EGF, TGFα and PDGF, and activate a downstream cascade consisting of RAS, RAF, KSR and the MAP kinase cascade (reviewed by Downward, 1995). Similarly, a higher order receptor, consisting of two type I subunits and two type II subunits is formed in response to TGFß that results in the activation of the receptors' cytoplasmic serine/threonine kinase domains that signal through MAD like molecules. However, much less is known about the roles and activities of receptor-kinase mediated signaling in plants.

Mutational analysis has uncovered several receptor kinases involved in plant development and pathogen defense. The *CLAVATA1* (*CLV1*) gene, which regulates apical and floral meristem development in *Arabidopsis* (Leyser and Furner, 1992; Clark et al., 1993), encodes a protein with a potential extracellular domain consisting of 21 tandem leucine-rich repeats followed by a transmembrane domain and putative cytoplasmic serine/threonine kinase domain (Clark et al., 1997). Leucine-rich repeats (LRRs) are found in a variety of proteins, both cytoplasmic and extracellular, including several animal hormone receptors and the Drosophila Toll and Chaoptin proteins. The CLV1 kinase domain autophosphorylates on serine residues, presumably by an intermolecular mechanism (Williams et al., 1997), suggesting that the active receptor is a dimer/multimer. This is similar to results for the RLK5 LRR-kinase of unknown function (Walker, 1993; Horn and Walker, 1994). However, downstream target proteins have yet to be identified, but the protein phosphatase containing KAPP protein might be a negative regulator of several receptor-kinase pathways (Stone et al., 1994; Braun et al., 1997; Williams et al., 1997). The *Arabidopsis ERECTA* gene encodes a protein similar to *CLV1* that controls

stem and floral organ length (Torii et al., 1996) and the *BRI1* gene encodes an LRR-kinase that likely is a plant steroid receptor (Li and Chory, 1997). In rice, the *Xa21* LRR-kinase has been implicated in pathogen recognition (Song et al., 1995).

Expression patterns can frequently suggest a function. The *CLV1* gene is expressed in the subepidermal regions of apical and young floral meristems, but is excluded from developing organ primordia (Clark et al., 1997), suggesting that CLV1 regulates cell proliferation of meristematic cells. However, very few other plant receptor-kinase gene expression patterns have been reported. To gain a sense of the possible roles of LRR-kinase during plant development, we have isolated two new genes with sequence similarity to *CLV1* and investigated the expression patterns of these genes and two other genes *RLK5* and *ERECTA*.

Results

Isolation of *CLAVATA1-LIKE* Genes

In an attempt to identify additional *Arabidopsis* genes encoding kinases, we used the portion of the *CLV1* gene encoding the kinase domain as a heterologous probe to screen a flower cDNA library (Weigel et al., 1992). Individual clones that hybridize to the *CLV1* gene under low stringency conditions but not at high stringency conditions were isolated. A total of two new genes were isolated and called *CLL2* (*CLAVATA1-LIKE 2*), and *CLL4*. The cDNA clones were sequenced in their entirety. Neither the *CLL2* nor *CLL4* cDNA were full length, but the entire *CLL4* sequence has recently been determined by the Kazusa DNA Research Institute while sequencing BAC clones (www.kazusa.or.jp/KDRI/kazusa_home-e.html). The *CLL2* gene has not been completely sequenced yet.

Database searches show that there are dozens of putative LRR-kinases in the Arabidopsis genome. It is hard to predict an exact number as many of the genes are from ESTs, BAC ends and genomic sequence and they have yet to all be combined. For further sequence analysis, we have included three additional LRR-kinase genes (ATF1C12, AC003981 and F21M12) recently identified as part of the Arabidopsis Genome Initiative.

Gene Structures, Sequence and Map Positions

LRR-kinases can have different genomic and molecular structures. For example, ER has 20 LRRs, TMK1 has 11 (Chang et al., 1992), BRI1 has 25 LRRs (Li and Chory, 1997), F21M12 has 19 and all the others, with the exception of CLL2 all the others here have 21 LRR (Figure 1A). BRI1 also has an island of 70 amino acids between the 21st and 22nd LRRs that may be functionally important (Li and Chory, 1997). The number of introns also varies among LLR-kinase encoding genes (Figure 1B). With the exception of *BRI1*, AC003981 and F12M12, all have an intron in box VIII of the kinase domain (Hanks and Quinn, 1991), and *ER* has additional introns in every LRR. The size of the intron also differs. For example, the intron in *CLV1* is 79 bases, in *CLL4* it is 109 bases, in *RLK5* it is 71 bases and in *CLL2* it is 267 bases.

We determined the relative chromosomal location of *CLL2* and *CLL4* by RFLP analysis (Chang et al., 1988). *CLL2* maps to the top arm of chromosome I (Figure 1B). No known visible mutants map near *CLL2*. *CLL4* maps near the bottom of chromosome V (Figure 1B). Interestingly, the *REVOLUTA* and *MGOUN1* genes map close to *CLL4*.

To better understand the possible relationships between these LRR-kinases, we constructed a dendogram using the kinase domains of all the genes in Figure 1A. CLV1, CLL4 and ATF1C12 group together, RLK5 and CLL2 group together, and ER and BRI1 form a group (Figure 1C).

CLL4 is a Functional Kinase

To see if CLL4, like CLV1 and RLK5, has intrinsic kinase activity, we expressed the CLL4 kinase domain in E. coli as a GST fusion protein. A fusion protein with the wild-type CLL4 kinase domain (GST-4KD) autophosphorylated in the presence Mg^{2+} and [γ-^{32}P]ATP; however, when the catalytic lysine was changed to glutamic acid (GST-$4KD_{K722E}$) the resulting protein failed to autophosphorylate (Figure 2).

Expression Analysis

To assess the possible roles of the LRR-kinases during plant growth and development, we determined the patterns of gene expression by in situ hybridization for *CLL2*, *CLL4*, *RLK5* and *ER*. For all cases, probes were derived from the end of the ORF and part of the 3' UTR that are quite different at the nucleotide level from each other and other LRR-kinases.

CLL4 is expressed on the flanks of the shoot apical meristem and in very young floral meristems. Later during flower development, *CLL4* mRNA is detected in stamen and carpel primordia (Figure 3A-B). *CLL4* mRNA is not detected in mature flowers. This is a pattern almost complementary to the expression of *CLV1* in the shoot apical meristem (Clark et al., 1997). *CLL4* is also expressed down the stem in the provascular tissue.

CLL2 is expressed in the tapetum, the cells lining the pollen sacs in the stamen (Figure 3C). *RLK5* is expressed in the putative abscission zone where the organs of the mature flower will fall off (Figure 3D). *ER* has a much wider expression domain. It is expressed throughout the shoot apical meristem and young floral meristems (Figure 4A). Later in flower development, *ER* is expressed in the carpels and petals coincident with the time that those organs are expanding, presumably due to cell expansion (Figure 4B-D).

Discussion

Do All LRR-Kinases Regulate Common Functions?

One possibility is that different LRR-kinase control a common cellular process. For example, do all LRR-kinases regulate aspects of cell proliferation? It is becoming clear that the answer to that question is "No." Whereas *CLV1* may have roles in regulating meristematic cell proliferation, the defects in *bri1* are clearly associated with cell expansion and *ER* may play a role in cell expansion. Furthermore, *RLK5* and *CLL2* are expressed in cells that have stopped dividing. Unfortunately, neither *CLL2* nor *RLK5* map near any known visible mutants, so further understanding of the gene family may require a reverse genetic approach.

A more appropriate question may be, "Have LRR-kinases with similar sequence assumed similar roles?" Using the kinase domains, CLV1 and CLL4 group together and both genes are expressed within regions of the shoot apical and floral meristems. So these two closely related genes may be regulating meristematic cells divisions. CLL2 and RLK5 have very similar amino acid sequences and both are expressed in the cells that die as part of normal floral development. Therefore, this family may regulate plant cell death. The ER and BRI1 kinase sequences are similar and both may affect cell expansion.

Role of *CLL4*?

35S::*KAPP* and 35S::CKD_{K720E}, a catalytically inactive form of the CLV1 kinase domain, have floral organ defects similar to the *clavata1 superman* double mutant phenotype (data not shown). Since KAPP functions as a negative regulator LRR-kinases and CKD_{K720E} likely acts as a dominant negative, there is likely an LRR-kinase that functions to regulate third whorl cell proliferation in a way similar to the *SUPERMAN* gene. Could *CLL4* be

that gene? It is expressed in stamen primordia at the same time as *SUPERMAN* (Sakai et al., 1995), but it is also expressed in other cells. One way to test this idea is to see the phenotype of plants carrying a 35S::*4KD*$_{K722E}$.

Furthermore, *revoluta* and *mgoun1* map near *CLL4* on the bottom of chromosome 5. *revoluta* mutants have altered leaf shape, floral organ number and the shoot apical meristem terminates early (Talbert et al., 1995). It is thought that the wild-type *REVOLUTA* gene acts to reduce the number of cell divisions in the leaves and promote growth of the shoot and floral meristems. Mutations in the *MGOUN1* gene result in plants that have enlarged shoot meristems and reduced numbers of leaves and floral organs (Laufs et al., 1998). *MGOUN1* may promote organ initiation on the flanks of the shoot meristem. It will be interesting the sequence *CLL4* from these mutants.

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