Chapter 3. Functional analysis of five CLE proteins by reverse genetics

3.1 Introduction

A major class of transmembrane receptors in plants is made up of receptor-like kinases, or RLKs. The *Arabidopsis thaliana* genome contains over 400 genes encoding these receptors, which are subdivided into smaller groups based on motifs in the extracellular domain. Slightly over half of the RLK family is made up of receptors with leucine-rich repeats (LRRs) in their extracellular portion (Shiu and Bleecker, 2001). LRR-RLKs have been shown to be instrumental in a number of developmental processes, including hormone response, shoot architecture, and floral organ abscission (Jinn et al., 2000; Li and Chory, 1997; Torii et al., 1996).

One of the first LRR-RLKs to be functionally characterized was *CLAVATA1* (*CLV1*), a receptor required to control cell proliferation in shoot and floral meristems (Clark et al., 1993; Clark et al., 1997; Jinn et al., 2000). Loss-of-function *clv1* alleles give rise to plants with enlarged, fasciated shoot meristems and flowers with increased organ number (Clark et al., 1993). Similar phenotypes are observable in *clv3* loss-of-function mutants (Figure 3.1, B). Since *CLV3* encodes small secreted protein of 96 amino acids (Fletcher et al., 1999; Rojo et al., 2002), it is considered a likely candidate for the CLV1 ligand. The predicted ligand-receptor relationship is supported by several pieces of genetic data. First, plants trans-heterozygous for mutant alleles of *clv1* and *clv3* show a *clv* phenotype, a result which in some situations points to physical interaction (direct or indirect) between the two proteins (Clark et al., 1995). Second,

constitutive overexpression of *CLV3* causes meristem termination in wild-type plants (Figure 3.1, C), but not in the absence of CLV1 activity, suggesting that CLV1 functions downstream of CLV3 (Fletcher et al., 1999). Third, *CLV3* expression in the stem cells partially overlaps with *CLV1* expression. Fourth, CLV3 can act non-cell-autonomously in the floral meristem (Fletcher et al., 1999; Lenhard and Laux, 2003; Rojo et al., 2002).

In addition to CLV1 and CLV3, a third protein may function as part of this signaling complex. Loss-of-function mutations in *CLV2* give rise to a weak *clavata*-like phenotype (Kayes and Clark, 1998). *CLV2* encodes an LRR-containing receptor-like protein, which lacks the kinase domain present in LRR-RLKs (Jeong et al., 1999). Although there is no biochemical evidence for the presence of CLV2 in this complex, work on the brassinolide (BL) receptor BRI1 has indicated that LRR-RLKs can indeed function as heterodimers. BRI1 is known to bind BAK1; both are RLKs and contain 25 and 5 LRRs, respectively (Li et al., 2002; Li and Chory, 1997).

There are very few known ligands that act on LRR-RLKs (all but five of the LRR-RLKs which have been functionally characterized are "orphan" receptors). They include hormones (BL, systemin [in tomato], and phytosulfokine) and exogenous proteins found in pathogens (flagellin, and an epitope from the rice pathogen *Xanthomonas oryzae*) (Gomez-Gomez and Boller, 2000; He et al., 2000; Matsubayashi et al., 2002; Montoya et al., 2002; Song et al., 1995). Phytosulfokine and systemin are small peptides (5 and 18 amino acids, respectively). CLV3 is therefore the largest endogenous protein believed to signal through an LRR-RLK.

Work in the McCormick lab has resulted in the characterization of a family of genes similar to *CLV3*, termed the *CLV3/ESR* (*CLE*) genes. There are 26 such genes in *Arabidopsis* (including *CLV3*), and additional genes have been identified in other plant species (maize, rice, soybean, tomato) but not in animals. Each of the *CLE* genes encodes a small protein predicted to be secreted or membrane-bound. All of these proteins also share a small C-terminal conserved domain of 15 amino acids, including an invariant histidine and arginine and a highly conserved glycine (Cock and McCormick, 2001). Subsequent work by Sharma and Fletcher has indicated that all but one of the 26 CLE genes in *Arabidopsis* are expressed at sufficient levels for detection by RT-PCR. In addition, their expression is tissue-specific. When fused with GFP and transiently expressed in leek epidermal cells, all three CLE genes tested were localized to the membrane or extracellular space, as predicted by their protein sequences (Sharma et al., 2003).

Recent studies of *CLE19* and *CLE40* have indicated that multiple CLE proteins may be capable of activating a restricted number of pathways, notably the CLV1 pathway in the shoot, and a potentially related (but not identical) pathway in the root. For example, root-specific overexpression of *CLE19* leads to a gradual reduction in cell number in the root meristem, and an extragenic suppressor of this phenotype has a *clv*-like effect on carpel number (Casamitjana-Martinez et al., 2003). No loss-of-function phenotype has been reported for *CLE19*, however. Also, mention of the widespread *CLE19* expression pattern and unpublished data alluding to additional, non-root phenotypes in *35S::BnLLP1* (the tomato ortholog of *CLE19*) would imply that CLE19 is just as likely to function in other tissues besides the root (Casamitjana-Martinez et al., 2003).

The data for CLE40 suggest that this protein is able to act both in the shoot and the root to limit cell division, and that its signaling capabilities in the shoot are CLV1-dependent. It was also noted in this study that *CLV3*, while not normally expressed in the root, can cause the same root meristem defect (when overexpressed) as *CLE40*. The only phenotype in the single loss-of-function allele of *CLE40* is a increased waving of the primary root, with no observable defect in the structure of the root meristem itself (Hobe et al., 2003).

These experimental data for CLE19 and CLE40 point to the possibility that multiple CLE proteins may be able to bind CLV1 and/or a CLV1-like receptor in the root meristem. A lack of meristematic phenotypes (in the shoot or the root) in *cle40* loss-of-function mutants indicates that a) the overexpression phenotype does not represent the endogenous function of CLE40 or that b) loss of CLE40 activity in the shoot and/or root may be more than adequately compensated for by CLV3. This second possibility has been tested: the *cle40*; *clv3-2* double mutant is indistinguishable from *clv3-2* (Hobe et al., 2003).

In this study, we have used reverse genetics to investigate the functions of five additional *CLE* genes: *CLE11*, *12*, *13*, *16*, and *17*. *CLE11*, *12*, and *13* are of particular interest because their sequences align closely with that of *CLV3* (see Figure 1D and Sharma et al., 2003). We show that CLE11 and CLE12 are capable of signaling through CLV1/CLV2 when overexpressed. Expression analysis and double-stranded RNA interference (dsRNAi) experiments indicate, however, that this phenomenon is likely not representative of the endogenous function of CLE11 and CLE12.

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CLV3   MDSKSFVLLLLLFCFLFLHDASD
CLE11  MTKQPKPCSFLFHISLLSALFVFLLISFAFTTSYKLKSGIN
CLE12  MLRISSSSSMALKFSQILFIVLWLSLFFLLLHHLYSLNFRRLYSLNAVEPSLLKQHYRSY
CLE13  MATTRVSHVLGFLLWISLLIFVSIGLFGNFSSKPINPFPSPVITLPALYY
CLE16  MEACSRKRRRRRAYTTSTTGYAAVFFCGIFVFAQFGISSSALFAP
CLE17  MTMCFFLFFFVFYVS                    FQIVLSSSAS
CLE19  MLHLFILYAPYSLYINISILILFALLSNVAIYNNPAFAFLHIISPSNKQKQYLTKNRQMKIKGLMILASSLLIL
CLE40  MAAMKYKGSVFIILVI

CLV3   LTQAHAHVQGLSNRKMMMMKMESEWVGANGEAEKAKTKGLGLHEELRTVPSGPDPLHHHVNPPRQPRNNFQLP
CLE11  SLGHKRILASNFDFTPFLKNKDRTQRQRQSPSLTVKENGFWYNDEERVVPSGPNPLHH
CLE12  RLVSRKVLSDRFDFTPFHSRDNSRHNHRSGEQYDGDEIDPRYGVEKRRVPSGPNPLHH
CLE13  RPGRRALAVKTFDFTPFL KDLRRSNHRKALPAGGSEIDPRYGVEKRLVPSGPNPLHH
CLE16  DHYPSLPRKAGHFHEMASFQAPKATVSFTGQRREEENRDEVYKDDKRLVHTGPNPLHN
CLE17      VGYSRLHLVASPPPPPPRKALRYSTAPFRGPLSRDDIYGDDKRVVHTGPNPLHN
CLE19  AFIHQSESASMRSLLMNNGSYEEEEQVLKYDSMGTIANSSALDSKRVIPTGPNPLHNR
CLE40  LLLSSSLLAHSSSTKSFFWLGETQDTKAMKKEKKIDGGTANEVEERQVPTGSDPLHHKHIPFTP
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Figure 3.1 CLAVATA3, a protein required for proper meristem regulation, is the founding member of the *CLV3/ESR* (*CLE*) gene family.
A) L-*er* (wild type) B) *clv3-2*. Loss-of-function mutations in *clv1* and *clv3* result in meristem fasciation and the production of extra organs in the flowers, particularly in the central whorls.
C) *35S::CLV3* plants have defective apical and axial meristems, each of which terminates after producing 1–3 leaves.
D) Sequence alignment of selected CLE proteins. The conserved C-terminal region is underlined. Functional analyses of CLV3, CLE19, and CLE40 have been published elsewhere (see text); reverse genetics experiments for CLE11-13, 16, and 17 are presented in this work.

3.2 Results

The overexpression of *CLE* genes results in specific developmental defects

To begin to understand the possible functions for the *CLE* genes, we overexpressed *CLE11-13*, *16*, *17*, and *CLV3*, under the control of the constitutive CaMV 35S promoter. As has already been published, plants carrying *35S::CLV3* have early-terminating meristems, both apically and axially. Since each meristem only gives rise to 1–3 leaves, this lends the plant a bushy appearance. The rare flowers produced by *35S::CLV3* have a reduced number of stamens and no central gynoecium (Fletcher et al., 1999). The phenotype of *35S::CLE12* (Figure 3.2, B and F) is remarkably similar, except that a greater proportion of the T1 plants produce flowers. Transgenic lines of intermediate strength are able to recover sufficiently from meristem termination to set seed, albeit at reduced yields compared to wild type. These plants are shorter in stature than wild type (data not shown). All of the misexpression experiments which include *CLE12* were also performed with *CLE11*, with identical results. For the sake of brevity, only the results from *CLE12* are shown.

CLE13, though grouped tightly with *CLV3*, *CLE11*, and *CLE12* (Sharma et al., 2003), does not cause a discernible phenotype when overexpressed (Figure 3.2, C and G). We confirmed that *CLE13* is transcribed at high levels in these lines, but we cannot determine the level of CLE13 protein in the absence of an antibody or tagged CLE13. The *CLE13* transcript may be under strict translational control, such that overexpressing the gene does not actually lead to higher abundance of protein. Alternatively, the CLE13 protein may require post-translational modification. It is also possible that CLE13 is unable to bind and/or

activate the CLV1 receptor. If this last option were true, it would suggest that either the binding specificity of the CLE proteins does not reside in the conserved C-terminal domain, or that unique sequences in the non-conserved portion of the protein actually inhibit or repress the binding of CLE13 to CLV1.

CLE17 (data not shown) and *CLE16* (Figure 3.2, D and H) do not affect either shoot or floral meristems when overexpressed. The only defects are in leaf shape (slightly smaller and narrower than wild type) and stature (as in *35S::CLE11* and *35S::CLE12*). As of this writing, there is no receptor-like kinase which is known to be involved in leaf size, with the exception of BRI1 (Li and Chory, 1997). Neither loss-of-function mutants in *BRI1* nor overexpression lines cause a phenotype that resembles *35S::CLE16* and *35S::CLE17*. Instead, decreases and increases in BRI1 activity cause severe dwarfing and greatly increased cell elongation, respectively (Li and Chory, 1997; Wang et al., 2001).

CLE12 overexpression phenotype requires _CLV1_ and _CLV2_, but not _CLV3_

Since *CLE12* has a similar overexpression phenotype to *CLV3*, we investigated whether CLE12, like CLV3, acts through the CLV1/CLV2 receptor complex. Plants homozygous for loss-of-function mutations in *clv1*, *clv2*, and *clv3* and transgenic for *35S::CLE12* demonstrate that this is in fact the case. *clv1* and *clv2* mutations are epistatic to *35S::CLE12* (as they are to *35S::CLV3*), suggesting that the wild-type function of these genes is required for the overexpression phenotype (Figure 3.3, A-C). *35S::CLE12* is epistatic to the *clv3*-2 mutant phenotype (Figure 3.3, D), implying that CLE12 does not need to form a heterodimer or multimer with CLV3 in order to bind the CLV1 receptor.

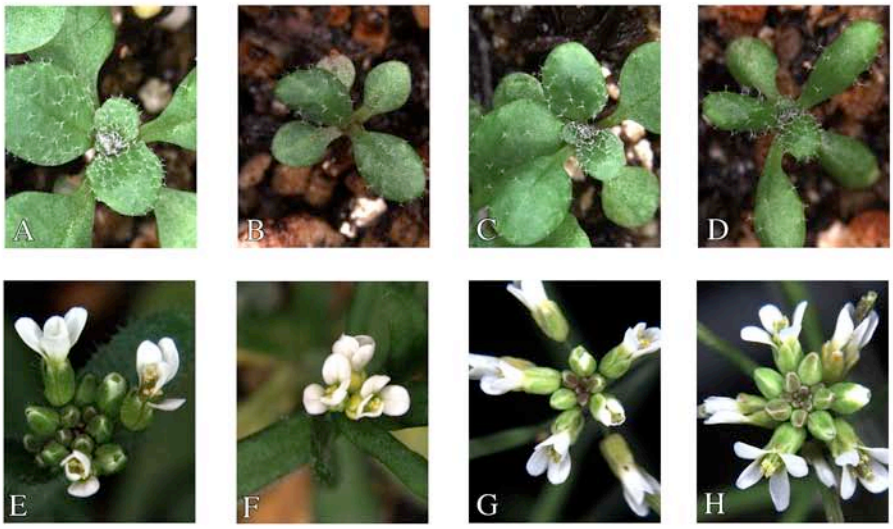


Figure 3.2 Overexpression of several *CLE* genes results in specific phenotypes.
A-D) Vegetative rosettes (17-day-old seedlings).
A) L-*er* (wild type).
B) *35S::CLE12*, like *35S::CLV3*, causes termination of the shoot apical meristem after 1, 2, or 3 true leaves. Axial meristems produce additional leaves, but never an ordered rosette.
C) *35S::CLE13* has no visible effect on vegetative growth.
D) Overexpression of *CLE16* results in smaller and narrower rosette leaves.
E-H) Inflorescences.
E) L-*er*.
F) Strong lines of *35S::CLE12* produce a limited number of flowers before the inflorescence meristem terminates. These flowers have a reduced number of stamens and no central gynoecium. Weaker *35S::CLE12* lines are able to recover sufficiently to make carpels and set seed (data not shown).
G) *35S::CLE13* is indistinguishable from wild type.
H) *35S::CLE16* shows no major defects in the flower, except a slight reduction in female fertility.

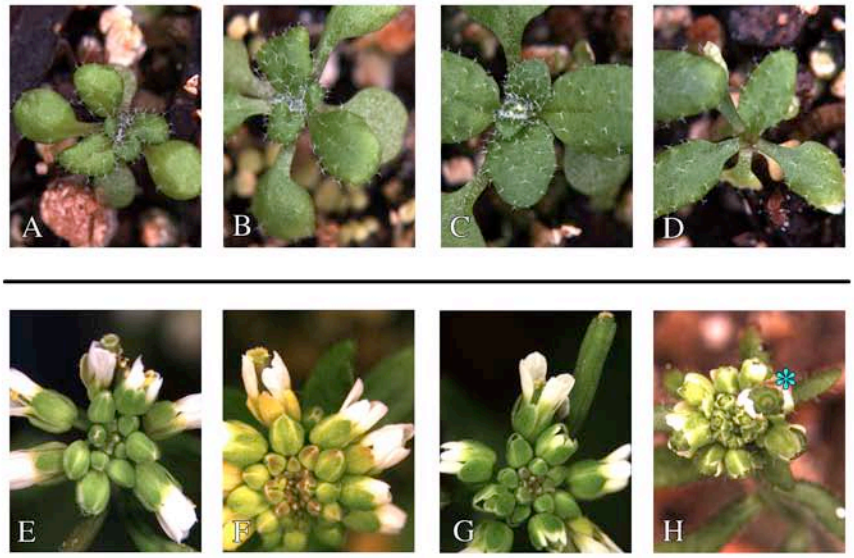


Figure 3.3 The *35S::CLE12* phenotype requires wild-type CLV1 activity, but not CLV3.
A) *35S::CLE12*; *clv1-1*
B) *35S::CLE12*; *clv1-6*
C) *35S::CLE12*; *clv2-1*
D) *35S::CLE12*; *clv3-2*. Note that *clv1-1*, *clv1-6*, and *clv2-1* are epistatic to *35S::CLE12*, but *35S::CLE12* is epistatic to *clv3-2*, as demonstrated by the terminated meristem in D.
E) *clv1-1* F) *35S::CLE12*; *clv1-1*
G) *clv1-6* H) *35S::CLE12*; *clv1-6*. Carpel number in *clv1-1* is unchanged with the addition of *35S::CLE12*, but is increased in *35S::CLE12*; *clv1-6* relative to *clv1-6* alone (see asterix in H).

There is a curious allele-specific effect of *35S::CLE12* on *clv1* mutants: the overexpression of *CLE12* enhances *clv1-6* dramatically in the flower (Figure 3.3, G and H), but has little influence on *clv1-1* and *clv1-4* (Figure 3.3, E and F). The *clv1-6* mutation is a one-base pair deletion which causes a frameshift and an early stop (causing much of the CLV1 kinase domain to be missing as a result), while the *clv1-1* and *clv1-4* alleles have missense mutations in the kinase domain and extracellular domain, respectively (Clark et al., 1997).

Physiological levels of CLE12 can substitute for CLV3

To determine whether the apparent function of these proteins was an artificial by-product of their overabundance, we set out to determine whether they could functionally replace CLV3 at physiologically relevant levels. For this experiment, we used the *CLV3* promoter to drive expression of *CLV3*, *CLE12*, and *CLE13* in a *clv3-2* mutant background. CLV3 rescues the *clv3-2* phenotype completely, while CLE12 shows nearly complete rescue (Figure 3.4, B and C, respectively). CLE13 has no effect.

The *CLE* genes are expressed in diverse but overlapping patterns in the inflorescence

To gain a better understanding of the endogenous functions of these proteins, we analyzed their expression patterns by generating beta-glucuronidase (GUS – EC# 3.2.1.31) reporter lines for each gene. We subcloned a given length of 5′ sequence for each gene (between 1.3 and 3.0 kb, depending on the proximity and orientation of the nearest 5′ gene). Transformants were stained for GUS activity to determine where each gene was expressed.

Figure 3.4 CLE12 can functionally replace CLV3 in the meristem.
A) *clv3-2* has enlarged shoot and floral meristems, resulting in fasciation and extra floral organs.
B) The expression of *CLV3* from its native promoter is sufficient to rescue the *clv3-2* phenotype.
C) *pCLV3::CLE12* shows partial rescue of *clv3-2*. Some fasciation is still evident.
D) CLE13, though highly similar to CLV3, CLE11, and CLE12 at the level of protein sequence, cannot rescue *clv3-2* when expressed from the *CLV3* promoter.

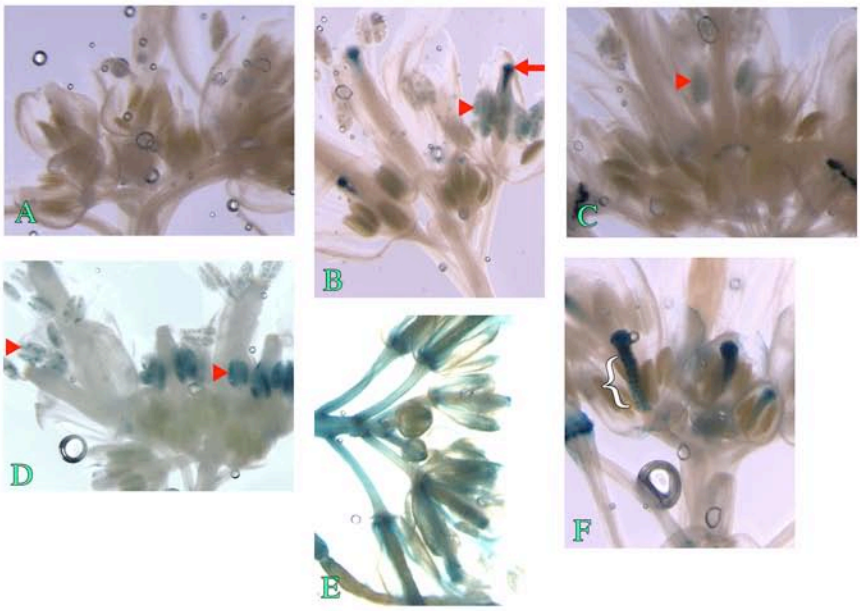


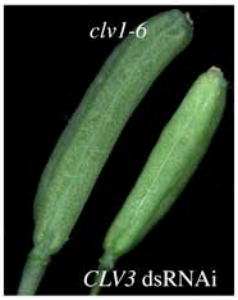
Figure 3.5 The *CLE* genes have discrete but overlapping expression patterns in the inflorescence.
A) L-*er* (wild type).
B) *pCLE11::GUS*. Note expression in developing pollen (arrowhead) and stigmae (arrow).
C) *pCLE12::GUS*. *CLE12* is expressed at low levels in stamens.
D) *pCLE13::GUS*, showing staining in stamens. The GUS activity is restricted to pollen in older stamens.
E) *pCLE16::GUS*
F) *pCLE17::GUS*. *CLE17* is expressed in stigmatic tissue and briefly in ovules (white bracket).

None of the five genes tested are expressed in the inflorescence meristem or floral meristems, suggesting that the overexpression phenotypes of *CLE11* and *CLE12* do not represent their true role *in vivo*. However, there are some specific yet overlapping expression patterns. In particular, *CLE11*, *12*, and *13* are expressed in stamens (Figure 3.5, B, C, and D), whereas *CLE11* and *CLE17* signal is found in stylar and early stigmatic tissue (Figure 3.5, B and F). *pCLE17::GUS* also shows temporally restricted but robust staining in ovules (Figure 3.5, F).

RNA interference of the *CLE* genes

We next set out to investigate whether these overexpression phenotypes (for CLE11, 12, 16, and 17) represented the endogenous function of these genes. Since there are no known point mutations or T-DNA insertions in any of these genes, we decided to use double-stranded RNA interference (dsRNAi). The constructs used contained the specific gene in the sense and antisense orientation, so that the resulting RNA would form a double-stranded structure. Such structures are known to be processed into siRNAs, which can destabilize the mRNA of the endogenous gene.

Plants carrying a *CLV3* dsRNAi construct had phenotypes similar to those of *clv3* and *clv1* loss-of-function mutants (Figure 3.6, A). Similar constructs made for the *CLE* genes had no visible effect under normal growing conditions (data not shown).



	T1	T2
35S::CLV3(AS)-intr-CLV3(S)	7/11	4/5
35S::CLE11(AS)-intr-CLE11(S)	0/26	0/10
35S::CLE12(AS)-intr-CLE12(S)	0/11	0/10
35S::CLE13(AS)-intr-CLE13(S)	0/14	0/10
35S::CLE16(AS)-intr-CLE16(S)	0/8	0/3
35S::CLE17(AS)-intr-CLE17(S)	0/14	0/9

Figure 3.6 Double-stranded RNA interference (dsRNAi) of *CLV3* has a *clv*-like phenotype, but no phenotypes are observed after dsRNAi of *CLE11-13*, *16*, or *17*.
The panel on the left shows green siliques from *clv1-6* and a typical *CLV3* dsRNAi line. The table at right lists the number of lines showing a phenotype over the total number of lines screened, in the T1 and T2 generations. For the *CLV3* dsRNAi T2 analysis, progeny of 3 strong lines, 1 intermediate line, and one wt-like line were grown.

3.3 Discussion

In the course of these experiments, we have gained insight the functions some of these CLE proteins are capable of performing. In particular, CLE11 and CLE12 can mimic CLV3 when overexpressed, and ectopic CLE16 and 17 activity can alter leaf shape. However, the total absence of RNA interference phenotypes leaves their true *in vivo* function a mystery. The failure of dsRNAi to cause mutant phenotypes for the *CLE* genes could suggest at least two possible scenarios. First, the reduction of function in one CLE protein could be compensated for by another. This is conceivable because (a) more than one *CLE* gene is expressed in a given tissue type (stamens, style) and (b) it is clear from this and other published work that many CLE proteins are capable of substituting for CLV3. A second scenario assumes that these five proteins are kept inactive under normal conditions, by mechanisms affecting such processes as post-translational modification, sub-cellular localization, or abundance of a co-factor and/or receptor.

Another intriguing aspect of this set of results is that the plants overexpressing these genes did not show defects in the tissues in which the genes are actually expressed. This phenomenon can be accounted for by at least two possible explanations. First, the CLE proteins may act under normal growth conditions to repress responses to external stimuli, such as heat, cold, salinity, or pathogen attack. In this situation, overexpression of a *CLE* gene will cause a phenotype (suppressed response) only in the presence of the appropriate external stimulus. Another explanation is similar to the second scenario described above for understanding the lack of RNAi phenotypes; namely, that

the activity of the CLE proteins is heavily regulated and restricted at the protein level, rather than at the level of transcription. One caveat is that this regulation, if at the level of protein stability or general receptor-binding activity, would have to be restricted to the tissues in which each gene is normally expressed; otherwise, there would be no overexpression phenotype at all. Stringent receptor-specific regulation would not necessarily have to be restricted to the tissues expressing each gene. If it was, however, aspects of the overexpression phenotype elsewhere in the plant could be explained by activation of the proper receptor in the wrong places. In other words, the effects of *35S::CLE16* and *35S::CLE17* on leaf shape may occur because the proper receptor(s) for these proteins are not under as tight regulation in leaves as they are in the tissues where *CLE16* and *CLE17* are normally expressed. The more conservative explanation for all of the overexpression phenotypes, however, is that the CLE proteins are binding receptors with which they do not interact under normal conditions—either because they are expressed ectopically, or because they are accumulating at such high levels that variables such as low binding affinity become irrelevant.

Although we have not deciphered much about the *in vivo* functions of these five proteins, the overexpression results may provide insight into the structural or sequence-level basis for the specificity of this family of genes. Particularly powerful analysis can be performed on the sequence requirements for successful activation of the CLV1 receptor. We know (from published work, as mentioned above) that CLE19 and 40 can activate CLV1, and now CLE11 and 12 can be added to the list. The overexpression phenotypes of CLE16 and 17 may represent useful negative results. Computational analysis, followed by judicious

domain-swapping and site-directed mutagenesis, should allow identification of exact domains or even residues required for binding to and activating CLV1.

One surprising result to emerge from these experiments is the curious enhancing effect of *35S::CLE11* and *35S::CLE12* on *clv1-6*, and the lack of an effect on the other alleles tested, including *clv1-1* and *clv1-4*. It has been suggested that although *clv1-6* is weaker than these other two alleles, it is closest to a true null allele, whereas the others are more complicated, possibly compromising the function of related receptors (Clark et al., 1997; Dievart et al., 2003). This would be particularly relevant if there is another LRR-RLK which can form functional heterodimers with CLV1, and which can also form homodimers with at least a limited CLV3-binding capability. For example, if region(s) missing in the *clv1-6* allele are required for robust dimerization, then the primary opportunity for CLV3 signaling would be through the homodimer of the other RLK. If the *clv1-1* and *clv1-4* versions of the protein are still able to form CLV3-binding heterodimers, but can transduce only a fraction of the normal signal, then the phenotype may be more severe.

It is not yet known whether *35S::CLV3* has the same effect on *clv1-6* (this experiment is underway), but if it does not (particularly if *35S::CLV3*; *clv1-6* resembles *35S::CLV3* in L-*er*), then the following model is possible, given the scenario described above: 1) CLE11 and CLE12 can bind both CLV1 and the other RLK, but can only produce signal through CLV1; 2) the overexpression of CLE11 or CLE12 in the *clv1-1* or *clv1-4* meristem has little effect on the CLV1/RLK heterodimers (particularly if the problem is in the propagation of the signal, rather than ligand binding); and 3) high levels of CLE11 or CLE12 in the *clv1-6* meristem bind the homodimers of the other RLK non-productively, out-

competing (by greater abundance) the endogenous, functional CLV3. This might be tested by co-overexpressing *CLV3* and *CLE12* (or *CLE11*) in *clv1-6*; if overexpressed CLE12 is drowning out effective signal from CLV3, adding high levels of CLV3 should negate the enhancing effect of *35S::CLE12* on *clv1-6*.

If, on the other hand, *35S::CLV3*; *clv1-6* resembles *35S::CLE12*; *clv1-6*, then the picture gets even more complicated. This result would raise the possibility that there are two opposing pathways, both of which can receive the CLV3 signal (and, in artificial situations, CLE11 or CLE12).

3.4 Materials and Methods

Plant growth conditions

Seeds were sown on a 4:3:2 mixture of potting soil, vermiculite, and perlite. Each flat was given sufficient water and kept at 4°C for 4 days for seed stratification. Plants were grown under 600 ft-candles of continuous cool white fluorescent light at a temperature varying between 17 and 21°C. Pests such as fungus gnats and aphids were kept under control by treatment with Gnatrol (20 ml in water) and granular Marathon (~ 4 g, post-germination).

Constructing vectors for transgenics – starting materials

PCR-amplified *CLE11-13*, *16*, and *17* products were cloned into pCR2.1 in at least one of three sets: complete coding sequence (plasmids **A-E**), coding sequence without stop codon (plasmids **A′**, **B′**, **D′**, and **E′** – not done for *CLE13*), and complete coding sequence with an extra BamHI site added at the 5′ end, for greater cloning flexibility (plasmids **F-I**; *CLE13* not included). A clone containing

the coding sequence of CLV3 in the BamHI site of pBS–SK was provided by F. Wellmer (plasmid **J**, orientation T7 5′ 3′ T3).

The *CLE* coding sequences were moved into pBS-SK to generate more restriction site options: plasmid **K** – BamHI fragment from F, orientation T7 5′ 3′ T3; plasmid **L** – EcoRV/HindIII fragment from B′, orientation T7 5′ 3′ T3; plasmid **M1** – EcoRI fragment from C, orientation T7 3′ 5′ T3; plasmid **M2** – EcoRI fragment from C, orientation T7 5′ 3′ T3; plasmid **N** – HindIII fragment from D′, orientation T7 5′ 3′ T3; plasmid **O** – EcoRV/HindIII fragment from E′, orientation T7 3′ 5′ T3; plasmid **P** – EcoRV/SpeI fragment from E′ into pBS-SK, orientation T7 5′ 3′ T3.

Some of the shuttle vectors included a modified pBJ36 (BaR), containing the 35S promoter from pHANNIBAL as well as the octopine synthase (OCS) terminator (plasmid **Q**, from J. Long), pBJ36 with the *CLV3* 5′ and 3′ sequences flanking the multiple cloning site (MCS) (plasmid **R**, from J. Long), and pRITA, which contains the GUS coding sequence following the MCS (plasmid **S**, from J. Long). Plasmid Q was modified for use as a basic RNAi vector by adding in the catalase intron (from pCATIN, J. Long) into the XbaI/BamHI site, to generate plasmid **T**.

We used pMLBART as a binary vector in all cases. Fragments were cloned into the NotI site of pMLBART; this is the last step for making all of the finished products detailed below.

Overexpression constructs

For *35S* overexpression constructs, we used pBJ36/*35S* (plasmid Q). The digests and source plasmids for each coding sequence are as follows: *CLE11*, BamHI, F;

CLE12, EcoRI, B; *CLE13*, SmaI/ClaI, M1; *CLE16*, EcoRI, D; *CLE17*, EcoRI, E; and *CLV3*, BamHI, J.

Expression from CLV3 promoter

All constructs with the *CLV3* promoter made use of plasmid R, one of the modified versions of pBJ36 (see above). The *pCLV3::CLE11*, *16*, *17*, and *pCLV3::CLV3* constructs were made with the BamHI fragment of plasmids F, H, I, and J, respectively. For *pCLV3::CLE13*, the SmaI/ClaI fragment from plasmid M1 was used, and for *pCLV3::CLE12*, we used the SmaI/XhoI fragment from the *35S::CLE12* construct.

dsRNA interference lines

For the *CLE11*, *12*, *16*, and *CLV3* dsRNAi lines, we ligated the BamHI fragment from plasmids F, G, H, and J (respectively) into plasmid T. Resulting clones with the correct orientation were subsequently linearized with XbaI and SalI. The XbaI/SalI fragments from plasmids K, L, N, and J were ligated into these vectors, to create antisense-linker-sense constructs.

For the *CLE13* and *17* dsRNAi lines, the SalI/SmaI fragments from plasmids M1 and O, respectively, were ligated into the XhoI/SmaI sites of plasmid T. The resulting clones were linearized with XbaI and SalI, and the XbaI/SalI fragment from plasmid M2 and P (respectively) were ligated into these vectors, again creating antisense-linker-sense constructs.

GUS reporter constructs

The 5' upstream region of each *CLE* gene was PCR-amplified and ligated into pCR2.1. For *pCLV3::CLE11*, the EcoRI fragment from the pCR2.1 clone was ligated into plasmid S, and resulting clones were checked for proper orientation. For *pCLV3::CLE12*, *13*, *16*, and *17*, the KpnI/XhoI fragment from the corresponding pCR2.1 clone was ligated into plasmid S.

Plant transformations

Plasmids were transformed into the ASE strain of *Agrobacterium tumefaciens* (kan chlor), and L-*er* plants were transformed by the floral dip method (Clough and Bent, 1998). Seeds were sown directly on soil, and T1 plants were sprayed with BASTA several times, starting about 6 days after germination.

GUS staining

Tissues were fixed in ice-cold 90% acetone for 30 minutes, then washed with rinse solution (34 mM Na_2HPO_4, 15.8 mM NaH_2PO_4, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4FE(CN)_6$, 0.5% Triton X-100). Rinse solution was replaced with a small amount of fresh solution (just enough to cover tissues) to which X-Gluc was added to a concentration of 2 mM (X-Gluc can be purchased as a powder and dissolved in DMF—100 mg in 1.92 ml to make a 100 mM stock solution). Tissues were vacuum infiltrated to draw solution into cells, then incubated in the dark at 37°C overnight, or until strong staining appeared. Tissues were then washed in 50% ethanol and cleared in 75% ethanol. Individual inflorescences and seedlings were mounted in single-depression slides in 50% glycerol, then photographed.

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3.6 References

Benjamins, R., Quint, A., Weijers, D., Hooykaas, P. and Offringa, R. (2001). The PINOID protein kinase regulates organ development in *Arabidopsis* by enhancing polar auxin transport. *Development* **128**, 4057-4067.

Casamitjana-Martinez, E., Hofhuis, H. F., Xu, J., Liu, C. M., Heidstra, R. and Scheres, B. (2003). Root-specific *CLE19* overexpression and the *sol1/2* suppressors implicate a CLV-like pathway in the control of *Arabidopsis* root meristem maintenance. *Current Biology* **13**, 1435-1441.

Clark, S. E., Running, M. P. and Meyerowitz, E. M. (1993). *CLAVATA1*, a regulator of meristem and flower development in *Arabidopsis*. *Development* **119**, 397-418.

Clark, S. E., Running, M. P. and Meyerowitz, E. M. (1995). *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. *Development* **121**, 2057-2067.

Clark, S. E., Williams, R. W. and Meyerowitz, E. M. (1997). The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. *Cell* **89**, 575-585.

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* **16**, 735-743.

Cock, J. M. and McCormick, S. (2001). A large family of genes that share homology with *CLAVATA3*. *Plant Physiology* **126**, 939-942.

Dievart, A., Dalal, M., Tax, F. E., Lacey, A. D., Huttly, A., Li, J. M. and Clark, S. E. (2003). *CLAVATA1* dominant-negative alleles reveal functional overlap between multiple receptor kinases that regulate meristem and organ development. *Plant Cell* **15**, 1198-1211.

Fletcher, L. C., Brand, U., Running, M. P., Simon, R. and Meyerowitz, E. M. (1999). Signaling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems. *Science* **283**, 1911-1914.

Gomez-Gomez, L. and Boller, T. (2000). FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Molecular Cell* **5**, 1003-1011.

He, Z. H., Wang, Z. Y., Li, J. M., Zhu, Q., Lamb, C., Ronald, P. and Chory, J. (2000). Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. *Science* **288**, 2360-2363.

Hobe, M., Muller, R., Grunewald, M., Brand, U. and Simon, R. (2003). Loss of CLE40, a protein functionally equivalent to the stem cell restricting signal CLV3, enhances root waving in *Arabidopsis*. *Development Genes and Evolution* **213**, 371-381.

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

Jinn, T. L., Stone, J. M. and Walker, J. C. (2000). HAESA, an *Arabidopsis* leucine-rich repeat receptor kinase, controls floral organ abscission. *Genes & Development* **14**, 108-117.

Kayes, J. M. and Clark, S. E. (1998). *CLAVATA2*, a regulator of meristem and organ development in *Arabidopsis*. *Development* **125**, 3843-3851.

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

Li, J., Wen, J. Q., Lease, K. A., Doke, J. T., Tax, F. E. and Walker, J. C. (2002). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell* **110**, 213-222.

Li, J. M. and Chory, J. (1997). A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* **90**, 929-938.

Matsubayashi, Y., Ogawa, M., Morita, A. and Sakagami, Y. (2002). An LRR receptor kinase involved in perception of a peptide plant hormone, phytosulfokine. *Science* **296**, 1470-1472.

Montoya, T., Nomura, T., Farrar, K., Kaneta, T., Yokota, T. and Bishop, G. J. (2002). Cloning the tomato *curl3* gene highlights the putative dual role of the leucine-rich repeat receptor kinase tBRI1/SR160 in plant steroid hormone and peptide hormone signaling. *Plant Cell* **14**, 3163-3176.

Rojo, E., Sharma, V. K., Kovaleva, V., Raikhel, N. V. and Fletcher, J. C. (2002). CLV3 is localized to the extracellular space, where it activates the *Arabidopsis CLAVATA* stem cell signaling pathway. *Plant Cell* **14**, 969-977.

Sharma, V. K., Ramirez, J. and Fletcher, J. C. (2003). The *Arabidopsis CLV3-like* (*CLE*) genes are expressed in diverse tissues and encode secreted proteins. *Plant Molecular Biology* **51**, 415-425.

Shiu, S. H. and Bleecker, A. B. (2001). Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 10763-10768.

Song, W. Y., Wang, G. L., Chen, L. L., Kim, H. S., Pi, L. Y., Holsten, T., Gardner, J., Wang, B., Zhai, W. X., Zhu, L. H. et al. (1995). A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. *Science* **270**, 1804-1806.

Torii, K. U., Mitsukawa, N., Oosumi, T., Matsuura, Y., Yokoyama, R., Whittier, R. F. and Komeda, Y. (1996). The *Arabidopsis ERECTA* gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. *Plant Cell* **8**, 735-746.

Wang, Z. Y., Seto, H., Fujioka, S., Yoshida, S. and Chory, J. (2001). BRI1 is a critical component of a plasma-membrane receptor for plant steroids. *Nature* **410**, 380-383.