

Functional Analyses of the CLAVATA2-Like Proteins and Their Domains That Contribute to CLAVATA2 Specificity^{1[C][W]}

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The Arabidopsis (*Arabidopsis thaliana*) CLAVATA2 (*CLV2*) gene encodes a leucine-rich repeat receptor-like protein (RLP) that is involved in controlling the stem cell population size in the shoot apical meristem. Our previous genome-wide functional analysis of 57 *AtRLP* genes revealed only a few phenotypes for mutant alleles, despite screening a wide range of growth and developmental stages and assaying sensitivity to various stress responses, including susceptibility toward pathogens. To gain further insight into the biological role of *AtRLPs*, in particular *CLV2*-related *AtRLP* genes, we tested their ability to complement the *clv2* mutant phenotype. We found that out of four close *CLV2* homologs tested, *AtRLP2* and *AtRLP12* could functionally complement the *clv2* mutant when expressed under the control of the *CLV2* promoter. This indicates that the functional specificity of these three genes is determined at the level of their transcriptional regulation. Single and double mutant combinations with impaired *AtRLP2* and/or *AtRLP12* did not show an aberrant phenotype, suggesting that other genes are redundant with these *CLV2*-like genes. To understand which protein domains are essential for *CLV2* function and which parts are interchangeable between related *CLV2*-like proteins, we performed domain-deletion and domain-swap experiments. These experiments revealed that *CLV2* remains functional without the island domain, whereas the C1 and C3 regions of the leucine-rich repeat domain are essential for functionality. Analysis of domain-swap constructs showed that the C3-G region of *CLV2* can be replaced by that of *AtRLP38*, although it could not complement the *clv2* mutant under control of the *CLV2* promoter. This suggests that the C3-G region is conserved among related *AtRLP* members, whereas the C1 domain may determine the functional specificity of *CLV2*.

Receptor-like proteins (RLPs) are cell surface receptors that typically consist of an extracellular Leu-rich repeat (eLRR) domain, a transmembrane domain, and a short cytoplasmic tail that lacks an obvious intracellular signal transduction domain apart from the putative endocytosis motif found in some members (Jones and Jones, 1997; Shiu and Bleeker, 2001; Kruijt et al., 2005; Wang et al., 2008). The first identified RLP gene was tomato (*Solanum lycopersicum*) *Cf-9*, a disease resistance gene that mediates resistance against strains

of the fungal leaf mold pathogen *Cladosporium fulvum* that carry the avirulence gene *Avr9* (Jones et al., 1994). The RLP disease resistance gene family comprises the tomato *Cf* and *Ve* genes that provide resistance against *C. fulvum* and *Verticillium* species, respectively, the *LeEIX* genes that encode receptors for the ethylene-inducible xylanase produced by *Trichoderma* biocontrol fungi, apple (*Malus domestica*) *HcrVf* genes that confer resistance to the scab fungus *Venturia inaequalis*, and an Arabidopsis (*Arabidopsis thaliana*) RLP gene (*AtRLP52*) that provides resistance against the powdery mildew pathogen *Erysiphe cichoracearum* (Kawchuk et al., 2001; Belfanti et al., 2004; Ron and Avni, 2004; Ramonell et al., 2005; Malnoy et al., 2008; Fradin et al., 2009). Furthermore, we recently demonstrated that Arabidopsis *AtRLP30*, and possibly *AtRLP18*, mediates nonhost resistance toward *Pseudomonas syringae* pv *phaseolicola* (Wang et al., 2008).

In addition to defense against pathogens, *AtRLP* genes also play roles in plant development. The developmental *AtRLP* genes comprise Arabidopsis *TOO MANY MOUTHS* (*TMM*; *AtRLP17*), which regulates stomatal distribution by controlling meristemoid formation as well as initiation of stomatal precursor cells (Yang and Sack, 1995; Nadeau and Sack, 2002). How-

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ever, probably the best studied developmental *RLP* gene is Arabidopsis *CLAVATA2* (*CLV2*; *AtRLP10*), which, like its maize (*Zea mays*) ortholog *FASCIATED EAR2* (*FEA2*), was found to be required for proper meristem development, stem cell specification, and organogenesis (Kayes and Clark, 1998; Jeong et al., 1999; Taguchi-Shiobara et al., 2001).

CLV2 is proposed to be part of a receptor complex containing the receptor-like kinase (RLK) *CLV1* (Clark et al., 1997; Trotochaud et al., 1999). RLKs are cell surface receptors that differ from RLPs because they contain a cytoplasmic kinase domain (Shiu and Bleecker, 2001). It has been proposed that *CLV1* and *CLV2* undergo a physical interaction to form a heterodimer that acts as a receptor complex for the extracellular peptide ligand *CLV3* (Clark et al., 1995; Trotochaud et al., 1999; Rojo et al., 2002; Ogawa et al., 2008). Upon *CLV3* perception by the *CLV1* ectodomain (Ogawa et al., 2008), the kinase domain of *CLV1* is thought to activate a yet uncharacterized downstream signaling cascade to repress expression of the stem cell-promoting transcription factor *WUSCHEL* (Lenhard and Laux, 2003) and is thus required to maintain the stem cell population (Rojo et al., 2002; Diévarit and Clark, 2004). Like *CLV2*, loss of function of *CLV1* and *CLV3* causes the progressive accumulation of undifferentiated stem cells, resulting in enlarged shoot apical meristems (SAMs) and increased floral organ numbers (Clark et al., 1993, 1995; Kayes and Clark, 1998). However, it has been shown that no additional organs develop in *clv2* flowers under short-day conditions, whereas the SAM remains enlarged, suggesting that the regulation of the flower meristem by *CLV2* is dependent on the physiological state of the plant (Kayes and Clark, 1998; Jeong and Clark, 2005). Alternatively, it is hypothesized that a yet unidentified *RLP* gene, specifically expressed under short days, is able to functionally compensate for the activity of *CLV2*. Furthermore, it was recently shown that *CLV2* acts together with the receptor kinase *CORYNE* (*CRN*), in parallel with *CLV1*, to perceive *CLV3* (Müller et al., 2008). *CRN* is identical to *Suppressor of Overexpression of LLP1-2* (*SOL2*), and loss of function of *SOL2* suppresses a short-root phenotype of transgenic plants constitutively overexpressing the *CLV3/ESR19* (*CLE19*) gene (Casamitjana-Martínez et al., 2003; Miwa et al., 2008). Since the *CRN* protein lacks a distinct extracellular domain, it was proposed that *CRN* and *CLV2* interact via their transmembrane domains to establish a functional receptor. Mutations in *CRN* cause stem cell proliferation, similar to *clv1*, *clv2*, and *clv3* mutants. However, *CRN* also shares additional functions during plant development with *CLV2*, including floral organ and root development (Miwa et al., 2008; Müller et al., 2008).

Typically, the amino acid sequences of RLPs are divided into the conserved domains A through G, with a putative signal peptide (A), a Cys-rich region (B), the eLRR domain (C), which is composed of two LRR regions (C1 and C3) that are separated by a non-

LRR island domain (C2), a Cys-rich spacer region (D), an acidic region (E), the transmembrane domain (F), and a short cytoplasmic tail (G; Jones and Jones, 1997). The eLRR domain, which is also found in the extracellular domain of RLKs, is proposed to play a versatile role in binding of ligands that can either be self (in development) or nonself (in pathogen resistance) molecules (Jones and Jones, 1997; Kobe and Kajava, 2001; Matsubayashi et al., 2002; Diévarit and Clark, 2004; Torii, 2004). Especially for the tomato Cf resistance proteins, extensive functional analysis of subdomains has been carried out. Domain-swap experiments between the tomato resistance proteins Cf-4 and Cf-9 revealed that recognition specificity resides in a number of residues at the solvent-exposed β -sheet of several distant LRRs in the C1 domain as well as in the number of LRRs (van der Hoorn et al., 2001; Wulff et al., 2001). Furthermore, conserved Trp and Cys pairs in the N-terminal LRR-flanking domain that are exposed at the putative concave inner surface of the Cf-9 protein, where also recognition specificity resides, were found to be important for Cf-9 function. Finally, many of the 22 putative N-linked glycosylation sites, especially those in the putative α -helices of the LRR modules, were found to contribute to Cf-9 activity (van der Hoorn et al., 2005). Several domain-swap experiments in which Cf-2/Cf-5 and Cf-2/Cf-9 chimeras were generated confirmed that recognition specificity resides in the C1 LRR domain (Seear and Dixon, 2003; Rivas et al., 2004).

Functional analysis of extracellular receptor domains has similarly been carried out using RLKs. The minimal hormone-binding domain for brassinosteroid hormones by Arabidopsis BRASSINOSTEROID INSENSITIVE1 (*BRI1*) comprises the 70-amino acid island domain (C2) and the C-terminal flanking LRR22 (Kinoshita et al., 2005). Finally, with a targeted Ala-scanning mutagenesis approach it was demonstrated that flagellin perception in Arabidopsis is dependent on a limited number of residues across LRR9 to LRR15 in the C1 domain of the RLK FLAGELLIN-SENSITIVE2 (*FLS2*) that binds bacterial flagellin (Dunning et al., 2007).

We have recently identified 57 *RLPs* in the Arabidopsis genome (*AtRLPs*) and reported on a genome-wide functional analysis of this gene family (Wang et al., 2008). Despite extensive analyses, few functions could be assigned to individual *AtRLP* members. It was suggested that the lack of phenotypes of individual T-DNA insertion mutants of *AtRLP* genes was caused by functional redundancy among gene family members (Wang et al., 2008). Nevertheless, an approach in which the expression of specific sets of multiple *AtRLP* genes was targeted by RNA interference also failed to uncover new biological functions for *AtRLP* genes (Ellendorff et al., 2008). To specifically investigate the role of functional redundancy among *AtRLP* genes, we studied the functionally characterized *CLV2* gene and its closest homologs, revealing that two *AtRLPs*, *AtRLP2* and *AtRLP12*, can replace

CLV2 function in a *clv2* mutant. Information about which domains of *CLV2*, and of its related homologs, are responsible for functionality and specificity is largely lacking. Therefore, a functional characterization of the *CLV2* protein was performed using deletion analyses and domain swaps between *CLV2* and a related RLP, *AtRLP38*, which was not able to complement *clv2* mutants. Our data indicate that the functional specialization among *CLV2*, *AtRLP2*, and *AtRLP12* can largely be attributed to differences in expression patterns. We further showed that the island domain of *CLV2* is dispensable for its function and that *CLV2* C3-G can be replaced by that of *AtRLP38*.

RESULTS

Identification of the *CLV2* Subfamily

In order to identify the *AtRLPs* that are the most related to *CLV2* (*AtRLP10*), the sequence similarity of the 56 remaining *AtRLPs* (Wang et al., 2008) with *CLV2* was determined. Although the *AtRLPs* have a low overall sequence similarity (Fritz-Laylin et al., 2005; Wang et al., 2008), domains C3 and D are conserved. For all *AtRLPs* that are similar in length (700–880 amino acids) and LRR number (19–25 LRRs) to *CLV2*, we selected the C3-to-D region for sequence alignment and constructed a phylogenetic tree to identify *CLV2*-related *AtRLPs* (Fig. 1; Supplemental Fig. S1). In this way, eight *CLV2*-like RLPs, namely *AtRLP2*, *AtRLP3*, *AtRLP11*, *AtRLP12*, *AtRLP30*, *AtRLP31*, *AtRLP37*, and *AtRLP38*, were identified as the closest *CLV2* homologs (Fig. 1). Four closely related protein pairs can be discriminated in the tree, namely *AtRLP2* and *AtRLP3*, *AtRLP11* and *AtRLP12*, *AtRLP30* and *AtRLP31*, and *AtRLP37* and *AtRLP38*, which, in all cases, are encoded by neighboring genes. It has previously been shown that of the 57 *AtRLPs*, 45 are predicted to contain a C2 island domain located between two eLRR blocks (C1 and C3; Wang et al., 2008). All eight *CLV2*-

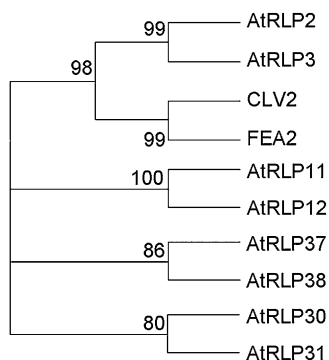


Figure 1. Phylogenetic tree of putative members of the *CLV2* subfamily and *FEA2*, the maize ortholog of *CLV2*. The tree was constructed for 100 bootstrap repetitions, and the numbers at the nodes indicate bootstrap support.

like *AtRLPs* carry an island domain that is variable in length and is followed by a C3 LRR domain of four LRRs (Supplemental Fig. S1). At the amino acid level, the eight *CLV2*-like RLPs exhibit a variable degree of conservation, ranging from 27% to 67% identity for the full-length proteins and 33% to 83% identity for the conserved part in the C3-to-D domain.

AtRLP2 and *AtRLP3* are the closest related to the *CLV2/FEA2* subclade (Fig. 1), leading to the hypothesis that especially those *AtRLPs* may have a biochemical function that is related to that of *CLV2*. One of the eight *CLV2*-like *AtRLPs*, *AtRLP30*, has recently been reported to play a role in nonhost resistance against *P. syringae* pv *phaseolicola* (Wang et al., 2008). No functions have been assigned to the other seven *CLV2*-like *AtRLPs* (Ellendorff et al., 2008; Wang et al., 2008).

AtRLP2 and *AtRLP12* Functionally Replace *CLV2* When Expressed under the Control of the *CLV2* Promoter

In order to test whether some of the eight *CLV2*-like *AtRLP* genes are functionally equivalent to *CLV2*, the ability of the *CLV2*-like *AtRLPs* to restore the phenotype of *clv2* mutants was examined. To this end, four selected *AtRLPs* (*AtRLP2*, *AtRLP12*, *AtRLP30*, and *AtRLP38*, one member of each protein pair; Fig. 1; Supplemental Fig. S1) were expressed under the control of the *CLV2* promoter (P_{CLV2}). As a control, similar constructs were made for *CLV2* and the functionally characterized *TMM* and tomato *Cf-9* genes. All constructs were used to transform the *clv2-3* mutant in the Landsberg *erecta* (*Ler*) genetic background (Kayes and Clark, 1998). A minimum of 20 T1 transformants for each construct were examined for complementation of the *clv2-3* mutant phenotype by analysis of their carpel numbers. As expected, transformation of the construct containing wild-type *CLV2* resulted in full complementation of *clv2-3*, as has been shown previously (Wang et al., 2008). Intriguingly, also *AtRLP2* and *AtRLP12* were able to fully rescue the *clv2-3* mutant, with a mean carpel number that is comparable to the wild type (Table I; Fig. 2, A–C). In the case of $P_{CLV2}::AtRLP2$, out of 30 independent transformants, 28 fully complemented *clv2*, with a mean carpel number 2.04 ± 0.20 . In the case of $AtRLP12$, out of 25 $P_{CLV2}::AtRLP12$ transgenic plants, 24 restored the *clv2-3* mutant to the wild type (Table I; Fig. 2, A–C). However, transformants expressing *AtRLP30*, *AtRLP38*, *Cf-9*, and *TMM* driven by the *CLV2* promoter showed no restoration of the carpel number phenotype (Table I; Fig. 2, A and B). In some cases, a few lines of transgenic plants expressing *AtRLP30*, *AtRLP38*, *Cf-9*, and *TMM* showed little degree of rescue by occasionally producing siliques with two or three carpels, but overall there was no statistical difference compared with *clv2-3*.

The *clv2* mutation also causes pedicel elongation (Kayes and Clark, 1998). We also assessed the pedicel length of transgenic plants carrying the different constructs. The pedicel length of *clv2* mutants is about 40% longer than that of wild-type plants, while the

Table 1. Carpel numbers of various mutants and transgenic lines generated in this study

Genotype	Construct	Daylength	Carpel No. (\pm SE)
<i>clv2-3</i>	$P_{CLV2}:CLV2$	Long days	2.05 \pm 0.09
<i>clv2-3</i>	$P_{CLV2}:AtRLP2$	Long days	2.04 \pm 0.20
<i>clv2-3</i>	$P_{CLV2}:AtRLP12$	Long days	2.06 \pm 0.20
<i>clv2-3</i>	$P_{CLV2}:AtRLP30$	Long days	3.60 \pm 0.36
<i>clv2-3</i>	$P_{CLV2}:AtRLP38$	Long days	3.62 \pm 0.32
<i>clv2-3</i>	$P_{CLV2}:TMM$	Long days	3.61 \pm 0.32
<i>clv2-3</i>	$P_{CLV2}:Cf-9$	Long days	3.59 \pm 0.35
<i>clv2-3</i>	Deletion construct Δ 1	Long days	2.06 \pm 0.28
<i>clv2-3</i>	Deletion construct Δ 2	Long days	3.86 \pm 0.46
<i>clv2-3</i>	Deletion construct Δ 3	Long days	3.75 \pm 0.52
<i>clv2-3</i>	Deletion construct Δ 4	Long days	4.03 \pm 0.41
<i>clv2-3</i>	$P_{CLV2}:CLV2RLP38-I$	Long days	3.86 \pm 0.46
<i>clv2-3</i>	$P_{CLV2}:CLV2RLP38-II$	Long days	2.10 \pm 0.21
<i>clv2-3</i>	$P_{CLV2}:CLV2RLP38-III$	Long days	3.85 \pm 0.40
<i>clv2-3</i>	$P_{CLV2}:CLV2RLP38-IV$	Long days	3.92 \pm 0.32
<i>clv2-3</i>	$P_{CLV2}:CLV2RLP38-V$	Long days	3.95 \pm 0.22
<i>clv2-3</i>	–	Long days	3.98 \pm 0.28
<i>clv2-7</i>	–	Long days	2.65 \pm 0.65
<i>atrlp2 clv2-7</i>	–	Long days	2.66 \pm 0.60
<i>atrlp2 atrlp12</i>	–	Long days	2.00 \pm 0.00
<i>atrlp12 clv2-7</i>	–	Long days	2.52 \pm 0.69
<i>clv2-3</i>	–	Short days	2.28 \pm 0.21
<i>clv2-7</i>	–	Short days	2.05 \pm 0.15
<i>atrlp2 clv2-7</i>	–	Short days	2.02 \pm 0.10
<i>atrlp2 atrlp12</i>	–	Short days	2.00 \pm 0.00
<i>atrlp12 clv2-7</i>	–	Short days	2.02 \pm 0.16

pedicel length of transgenic plants expressing $P_{CLV2}:AtRLP2$ and $P_{CLV2}:AtRLP12$ is very close to the pedicel length of wild-type Arabidopsis plants (Fig. 2D). However, the pedicel length of transgenic plants carrying *AtRLP30*, *AtRLP38*, *Cf-9*, and *TMM* is comparable to that of *clv2* (Fig. 2D), revealing that those genes cannot substitute the *CLV2* function in pedicel development.

We subsequently overexpressed *AtRLP2* and *AtRLP12* under the control of the cauliflower mosaic virus 35S promoter in wild-type Arabidopsis. The overall growth and appearance of *AtRLP2*- and *AtRLP12*-overexpressing plants were indistinguishable from wild-type plants grown under normal growth conditions (data not shown).

***AtRLP2* and *AtRLP12* Show a Distinct Expression Level with *CLV2* in the SAM but Overlapping Expression Patterns in Other Organs**

Although *AtRLP2* and *AtRLP12* were able to rescue the phenotype of the *clv2-3* mutant when expressed under the control of the *CLV2* promoter, *atrlp2* and *atrlp12* mutants do not show enlarged meristems or multicarpel phenotypes themselves (Wang et al., 2008). Therefore, we examined the expression patterns of *CLV2*-like genes in the SAM by querying a newly available expression map of SAM (Supplemental Fig. S2; Yadav et al., 2009). In general, very low expression of *AtRLP2*, *AtRLP3*, *AtRLP11*, *AtRLP12*, *AtRLP30*, and *AtRLP31* was detected in the shoot apex (Supplemental Fig. S2A; Yadav et al., 2009), while *CLV2* was found

to be relatively highly expressed (Supplemental Fig. S2A), despite the fact that expression is much lower than that of *CLV1* (Yadav et al., 2009). Indeed, the result is also confirmed by the analysis of public microarray data from Genevestigator (Supplemental Fig. S2A; Zimmermann et al., 2004). Altogether, the distinct expression level in the SAM of *CLV2* and other *CLV2*-like members suggested that the subfunctionalization of these *CLV2*-like genes has largely occurred from their promoter specificities and the resulting expression domain.

To gain a more comprehensive view of *CLV2*-like gene expression, a search of the Web-based microarray database Genevestigator was conducted (Zimmermann et al., 2004; Supplemental Fig. S2B). *AtRLP2* and *AtRLP3* showed the highest similarity in their profiles both in expression pattern and in expression level (Supplemental Fig. S2), leading to the hypothesis that these two paralogous genes possess conserved functions after duplication. *AtRLP11* and *AtRLP12* also share a similar overall expression profile, while *AtRLP11* shows a remarkably lower expression level than *AtRLP12* (Supplemental Fig. S2B). *CLV2* is expressed at a constitutive level in many tissues examined (Jeong et al., 1999) and is also confirmed by microarray analysis (Supplemental Fig. S2B). Furthermore, *CLV2* expression also correlated well with several other *CLV2*-like genes in some organs. *CLV2* and *AtRLP12* both exhibit a high expression levels in seedlings, roots, and later roots (Supplemental Fig. S2B). *AtRLP3*, *AtRLP31*, and *CLV2* display very similar

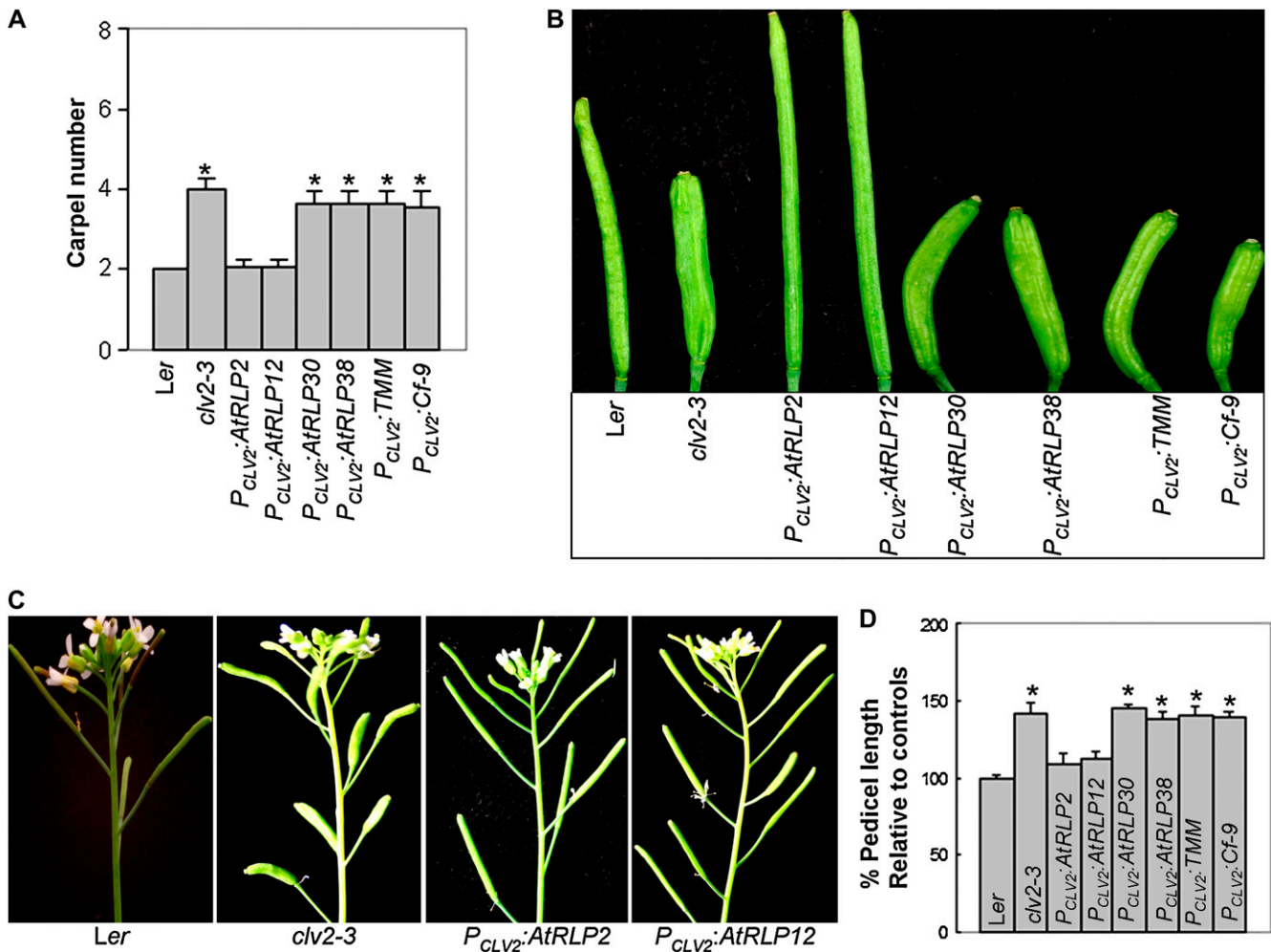


Figure 2. *AtRLP2* and *AtRLP12* complement the *clv2-3* mutant. **A**, Average number of carpels per flower for multiple independent transgenic lines of $P_{CLV2}:AtRLP2$, $P_{CLV2}:AtRLP12$, $P_{CLV2}:AtRLP30$, $P_{CLV2}:AtRLP38$, $P_{CLV2}:TMM$, $P_{CLV2}:Cf-9$, wild-type *Ler*, and the *clv2-3* mutant. For each construct, a minimum of 20 T1 transgenic plants with 30 siliques per plant were counted for each mean number. **B**, Representative siliques of wild-type *Ler*, *clv2-3*, $P_{CLV2}:AtRLP2$, $P_{CLV2}:AtRLP12$, $P_{CLV2}:AtRLP30$, $P_{CLV2}:AtRLP38$, $P_{CLV2}:TMM$, and $P_{CLV2}:Cf-9$ plants. **C**, Representative inflorescences of 3-week-old *Ler*, *clv2-3*, $P_{CLV2}:AtRLP2$, and $P_{CLV2}:AtRLP12$. **D**, Mean pedicel length of multiple independent transgenic lines of $P_{CLV2}:AtRLP2$, $P_{CLV2}:AtRLP12$, $P_{CLV2}:AtRLP30$, $P_{CLV2}:AtRLP38$, $P_{CLV2}:TMM$, $P_{CLV2}:Cf-9$, and the *clv2-3* mutant relative to the wild type. At least 50 pedicels for each genotype were measured. Asterisks in **A** and **D** indicate significant differences ($P < 0.01$) compared with the respective wild types. [See online article for color version of this figure.]

expression profiles in seedlings and leaves (Supplemental Fig. S2B). Given that genes functioning in similar processes often display similar expression patterns, we expected that *CLV2* may have overlapping functions with other members in those organs. Interestingly, a survey of the SAM expression map revealed that several *CLV2*-like genes exhibit expression in the shoot apex (Supplemental Fig. S3), while their functions in the regulation of SAM remain unclear.

Characterization of the *atrlp2*, *atrlp12*, and *clv2* Mutant Combinations

To reveal possible overlapping functions among *AtRLP2*, *AtRLP12*, and *CLV2* during plant develop-

ment, we generated combinations of double mutations for these genes. In a recent study, we characterized a new *clv2* allele in the Columbia (Col-0) ecotype, *atrlp10*, which exhibited similar phenotypes as reported for previously characterized *clv2* alleles that are in the *Ler* ecotype (Kayes and Clark, 1998; Jeong et al. 1999; Wang et al., 2008). In accordance with previous designation (Kayes and Clark, 1998; Müller et al., 2008), we renamed *atrlp10* as *clv2-7*. To avoid background effects, we crossed the *atrlp2* and *atrlp12* mutants with the *clv2-7* mutant (all in the Col-0 background) to generate the double mutants *atrlp2 clv2-7*, *atrlp12 clv2-7*, and *atrlp2 atrlp12*. The double mutants were further phenotypically characterized with respect to their overall growth and development.

In none of the combinations did the double mutants exhibit any additional phenotype. Specifically, the carpel number as well as the response of the double mutants to treatment with a synthetic CLV3p peptide that corresponds to the conserved CLE motif that is present in CLV3-like peptide ligands (Fiers et al., 2005) were tested. Similar to wild-type plants and single mutant lines, *atrlp2 atrlp12* double mutants did not show altered phenotypes with respect to either carpel number or CLE peptide treatment (Table I; Fig. 3). The carpel numbers of the double mutants that contained *clv2-7* (*atrlp2 clv2-7* and *atrlp12 clv2-7*) were similar to those of *clv2-7* (Table I; Fig. 3A). Furthermore, *atrlp2 clv2-7* and *atrlp12 clv2-7* double mutants failed to respond to treatment with the CLE peptides, resulting in normal long roots (Fig. 3, B and C), as is also the case for *clv2-7* single mutants. In an attempt to unravel the function of *AtRLP2* and *AtRLP3*, we made an RNA interference construct to target the expression of *AtRLP2* and *AtRLP3* simultaneously (Ellendorff et al., 2008). However, no developmental anomalies were observed for the RNA interference lines (data not shown).

It has been proposed that a yet unidentified RLP protein is able to functionally compensate the activity of CLV2 based on the fact that the floral meristem defects of the *clv2* mutant are suppressed under short-day conditions (Kayes and Clark, 1998; Jeong and Clark, 2005). Therefore, we tested whether the *atrlp2 clv2-7* and *atrlp12 clv2-7* double mutants developed additional floral organs under short-day conditions. For all mutants, the carpel number decreased to values similar to those of wild-type plants (Table I; Fig. 3A).

Deletion Analysis of the CLV2 Gene

The structure of RLP proteins is composed of distinct domains A to G (Jones and Jones, 1997; Fig. 4A; Supplemental Fig. S1). To test the contributions of various domains to CLV2 function, four deletion constructs were designed in which different domains of CLV2 were removed (Fig. 4A; constructs $\Delta 1$ – $\Delta 4$) based on the CLV2 protein organization. In the $\Delta 1$ construct, the C2 domain (the island domain) was removed, while in the $\Delta 2$ construct, LRR18 to LRR21, which compose the C3 domain, was removed. In the $\Delta 3$ construct, both the C2 and C3 domains were removed, while in the $\Delta 4$ construct, the C1 LRR domain (LRR1–LRR17) was removed (Fig. 4A). All mutant CLV2 genes, driven by the CLV2 promoter, were transformed to the *clv2-3* mutant to test their functionality. Reverse transcription-PCR analysis of multiple transgenic plants demonstrated the expression of the corresponding transgenes (Supplemental Fig. S4). Subsequently, a minimum of 25 transformants for each construct were examined for complementation of the *clv2-3* mutant phenotype by analysis of the carpel numbers. As expected, $P_{CLV2}:CLV2$ resulted in a complete restoration of the *clv2-3* mutant to a wild-type carpel number (Table I; Fig. 4). For deletion

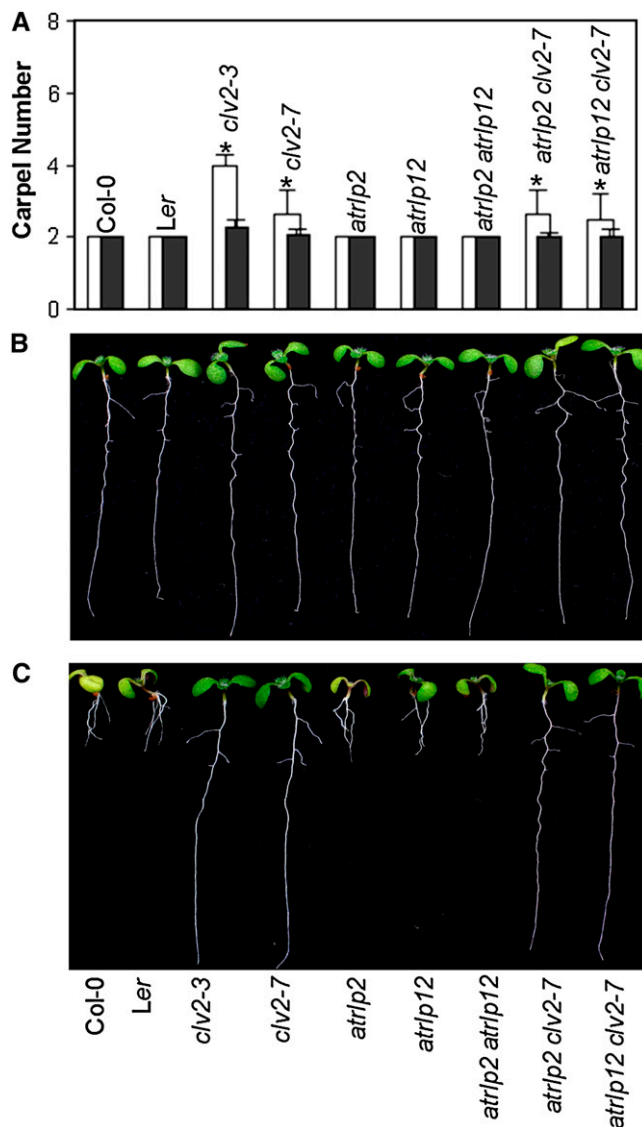


Figure 3. Phenotypic analysis of double mutants. A, Mean number of carpels per flower (\pm se) from plants grown under long-day conditions (white bars) and short-day conditions (black bars) of the double mutants *atrlp2 atrlp12*, *atrlp2 clv2-7*, and *atrlp12 clv2-7* with Col-0, *Ler*, *clv2-3*, and *clv2-7*. At least 100 siliques for each genotype were counted for the mean numbers. Asterisks indicate significant differences ($P < 0.01$) compared with the respective wild types. B and C, CLE peptide sensitivity assay, in which CLV3p peptide was added at a concentration of $10 \mu\text{M}$. Representative 8-d-old seedlings grown in the absence (B) and in the presence (C) of CLV3p peptide are shown. [See online article for color version of this figure.]

construct $\Delta 1$, 21 out of 22 T1 independent transgenic lines fully complemented the *clv2-3* mutant, exhibiting a mean carpel number of 2.06 ± 0.28 (Table I; Fig. 4). In contrast, deletion constructs $\Delta 2$, $\Delta 3$, and $\Delta 4$ could not complement the *clv2-3* mutant (Table I; Fig. 4), albeit some siliques of a few transgenic lines formed two to three carpels, thus resulting in a slightly decreased carpel number.

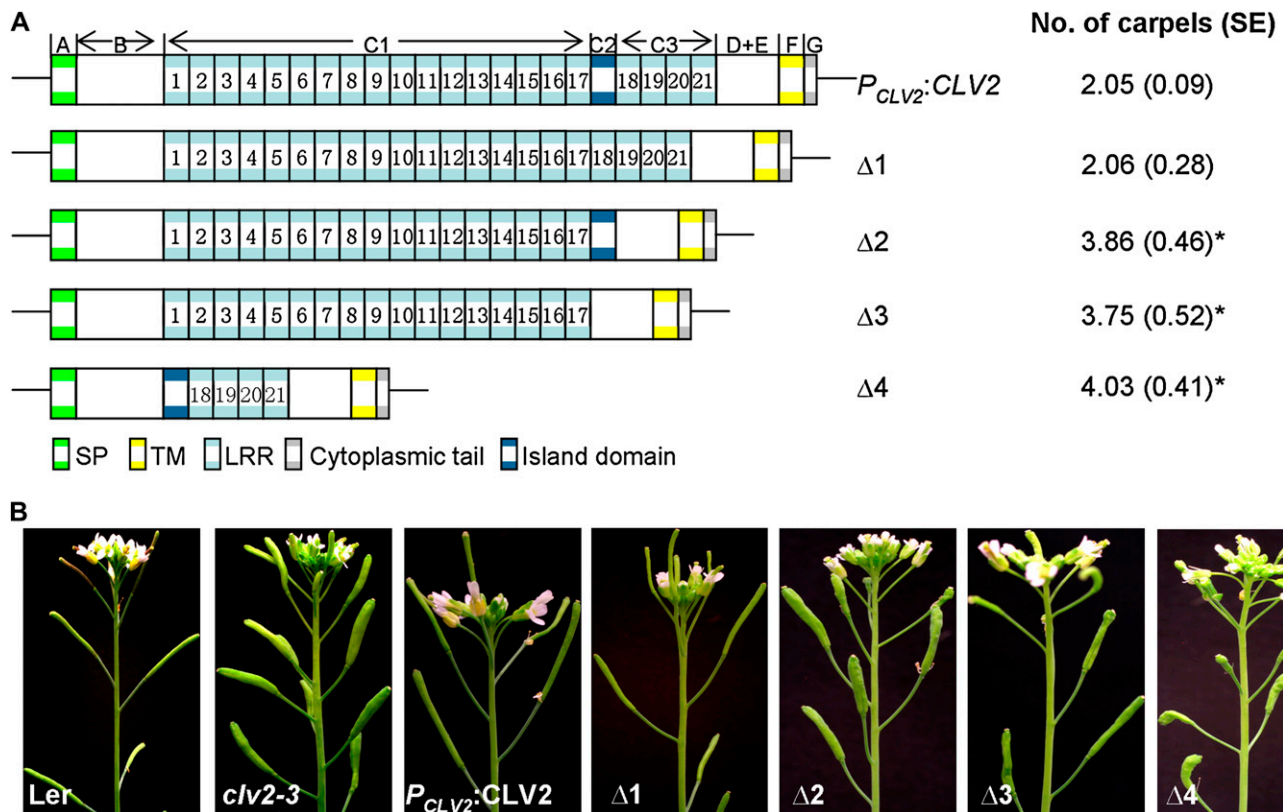


Figure 4. CLV2 deletion analysis. A, Schematic representation of the $P_{CLV2}:CLV2$ and four deletion constructs ($\Delta 1$ – $\Delta 4$) that were generated by removing the genomic sequence of the C2 island domain ($\Delta 1$), the genomic sequence of LRR18 to LRR21 that compose the C3 domain ($\Delta 2$), the genomic sequence of both the C2 and C3 domains ($\Delta 3$), or the genomic sequence of LRR1 to LRR17 that compose the C1 domain ($\Delta 4$). Lines indicate noncoding sequence, and boxes indicate coding sequence. The mean numbers of carpels per flower (\pm SE) of transgenic *clv2-3* plants expressing the different constructs are indicated on the right. For each construct, a minimum of 20 T1 transgenic plants with 30 siliques per plant were counted for the mean numbers. Asterisks indicate significant differences ($P < 0.01$) compared with the respective wild types. B, Inflorescences of wild-type (*Ler*), *clv2-3*, and transgenic *clv2-3* plants expressing the various constructs shown in A.

The C3-G Region of CLV2 Can Be Replaced by That of AtRLP38

AtRLP38 encodes one of the eight CLV2-like AtRLPs that share 42% overall sequence similarity and 28% identity with CLV2 (Wang et al., 2008; Fig. 1; Supplemental Figs. S1, S5, and S6). While *AtRLP38* contains 22 LRRs, CLV2 contains 21 LRRs (Fig. 1; Supplemental Figs. S1 and S6). As has been noted previously for other AtRLPs, sequence alignment of CLV2 and *AtRLP38* showed that the similarity is highest in the C3 LRR domain and the C-terminal region (Wang et al., 2008; Supplemental Fig. S5), and most variation is found in the N-terminal part of the C1 LRR domain. *AtRLP38* is not able to complement the *clv2-3* mutant when expressed under control of the *CLV2* promoter, suggesting that both *AtRLPs* have distinct functions (Fig. 2, A, B, and D). To further determine the requirement for individual CLV2 domains in regulating meristem development, we generated several chimeras between *CLV2* and *AtRLP38* (Fig. 5A), which were expressed under the control of the *CLV2* promoter in

clv2-3. Of these, only $P_{CLV2}:CLV2RLP38-II$ could complement the *clv2-3* mutant phenotype, as shown by restoration of the carpel number to the wild-type level (Table I; Fig. 5, B and C). Out of 28 T1 independent transgenic lines, six lines showed complete complementation, as shown by a mean carpel number of 2.10 ± 0.21 (Table I; Fig. 5, B and C). In this chimera, domains A to C2, including the large LRR region of the C1 domain and the island domain, were derived from CLV2, while the C3 to G domains, including the cytoplasmic tail, were derived from *AtRLP38* (Fig. 5A). Despite swapping the N-terminal domain of the CLV2 protein with the N-terminal domain of *AtRLP38*, the protein retained CLV2 biochemical activity (Fig. 5, B and C). No complementation of the *clv2-3* phenotype was observed for the other constructs ($P_{CLV2}:CLV2RLP38-I$, $-III$, $-IV$, and $-V$) in which the C1 domain of CLV2 was partially or completely exchanged with that of *AtRLP38* (Table I; Fig. 5), suggesting that the C1 domain is critical for CLV2 function. Occasionally, in a very few transgenic plants expressing the $P_{CLV2}:CLV2RLP38-I$, $P_{CLV2}:CLV2RLP38-III$, $P_{CLV2}:CLV2RLP38-IV$,

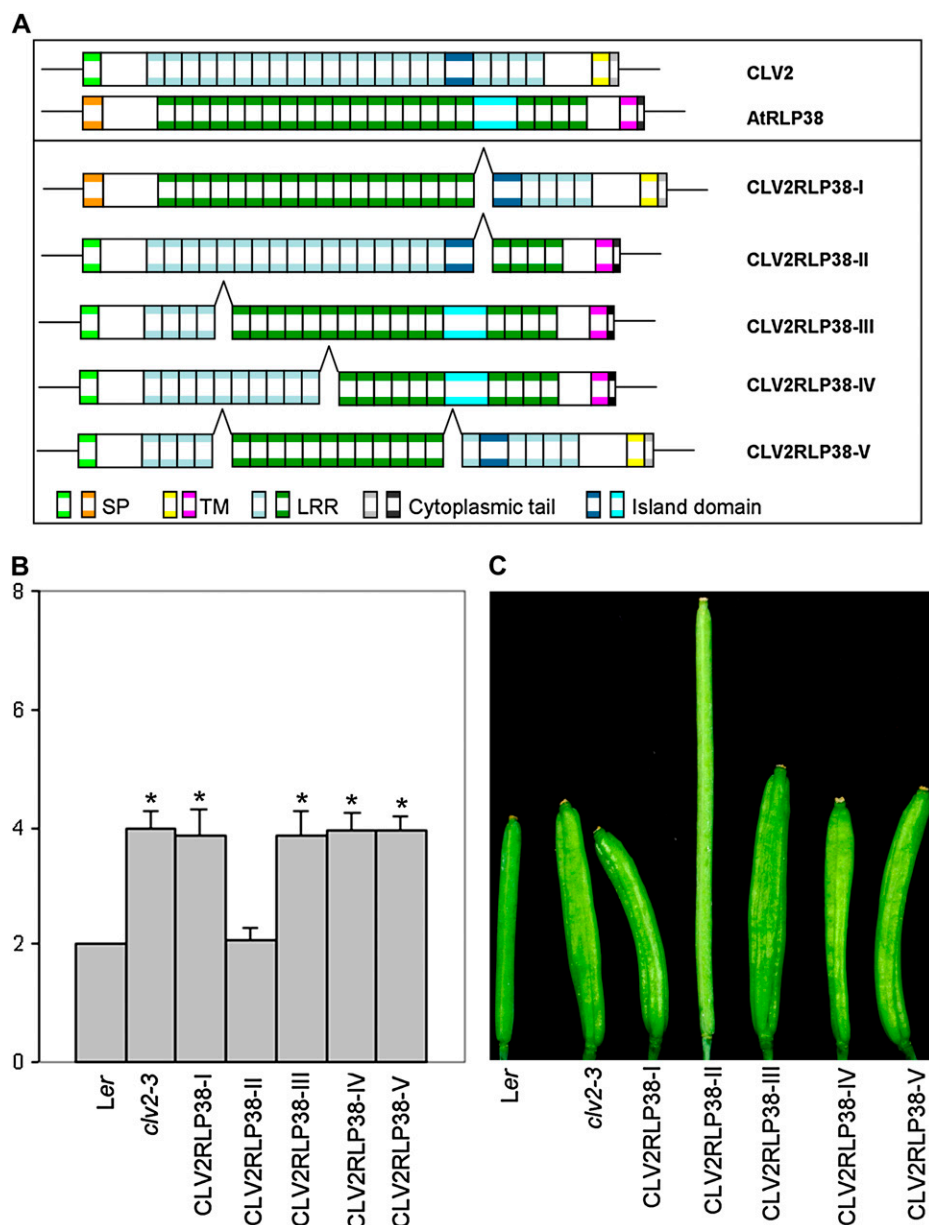


Figure 5. Analyses of domain swaps between CLV2 and AtRLP38. **A**, Schematic representation of the diverse chimeric proteins *CLV2RLP38-I*, *CLV2RLP38-II*, *CLV2RLP38-III*, *CLV2RLP38-IV*, and *CLV2RLP38-V*. The structures of various constructs are shown by different colors representing the distinct domains of the proteins. The junction between *CLV2* and *RLP38* in each chimeric construct is indicated by an open triangle. All constructs are driven by the *CLV2* promoter. **B** and **C**, Complementation results for expression of the *CLV2*-AtRLP38 chimeric receptors (*CLV2RLP38*) in *clv2-3*. **B**, The mean number of carpels per flower (\pm se) of multiple independent transgenic lines for *clv2-3* transformed with P_{CLV2} :*CLV2RLP38-I* to *-V*. The mean number of carpels for wild-type *Ler* and the *clv2-3* mutant are shown as controls for comparison. For each construct, a minimum of 20 T1 transgenic plants with 30 siliques per plant were counted for the mean numbers, while in the case of P_{CLV2} :*CLV2RLP38-II*, only the complete rescued transgenic lines were analyzed for the mean number. Asterisks indicate significant differences ($P < 0.01$) compared with the respective wild types. **C**, Comparisons of representative siliques from *Ler*, *clv2-3*, P_{CLV2} :*CLV2RLP38-I*, P_{CLV2} :*CLV2RLP38-II*, P_{CLV2} :*CLV2RLP38-III*, P_{CLV2} :*CLV2RLP38-IV*, and P_{CLV2} :*CLV2RLP38-V*.

and P_{CLV2} :*CLV2RLP38-V* chimeras, some siliques of the plants developed two or three carpels, suggesting that these chimeric constructs could incidentally complement the *clv2-3* mutation. None of the chimeric receptors showed any dominant-negative effects.

DISCUSSION

An initial genome-wide functional analysis of *AtRLP* gene T-DNA insertion mutants revealed only a few phenotypes, despite the screening of a wide range of conditions (Wang et al., 2008). This observation suggests a high degree of functional redundancy among the *AtRLP* family members. In this study, we first focused on *CLV2* and its closest homologs. A

subfamily consisting of *CLV2* and the eight most conserved *CLV2*-like *AtRLPs* was determined, and the functional conservation among the *CLV2*-like genes was tested by their ability to complement the *clv2-3* mutant. These experiments showed that, when expressed under control of the *CLV2* promoter, *AtRLP2* and *AtRLP12* were able to complement the developmental phenotypes of the *clv2-3* mutant, while the other tested *CLV2*-like *AtRLP* members were not. Our results suggested that the subfunctionalization of some *CLV2* subfamily members, at least among *CLV2*, *AtRLP2*, and *AtRLP12*, was primarily determined by the evolutionary divergence of their promoter specificities and expression patterns rather than by their protein-coding regions. A functional diversity

among closely related genes is, at least in part, due to diversification in gene expression.

None of the double mutant combinations showed additive phenotypic effects when compared with the *clv2-7* mutant allele. The observation that *AtRLP3* and *AtRLP11* are duplicated genes of *AtRLP2* and *AtRLP12*, respectively, suggests a similar function for these paralogues. In addition, the fact that *AtRLP2/AtRLP3* and *AtRLP11/AtRLP12* exhibit very similar overall expression profiles reinforces the hypothesis that they possess a conserved function. Therefore, the lack of additional phenotypes in the double mutants might be due to remaining activities of *AtRLP3* and *AtRLP11*. Consequently, different higher order mutant combinations, for instance, *AtRLP2/AtRLP3* and *AtRLP11/AtRLP12* mutants in combination with the *clv2* mutant, are needed to unravel the function of these *CLV2*-like genes.

We showed that *AtRLP30* and *AtRLP38* were unable to substitute the function of *CLV2* when expressed under control of the *CLV2* promoter, suggesting that they may have evolved toward distinct biological functions. Indeed, *AtRLP30* has been demonstrated to be involved in nonhost resistance toward the bacterial pathogen *P. syringae* pv *phaseolicola* (Wang et al., 2008). This suggests that the function of each gene in a gene family does not have to be correlated to the bioinformatic and phylogenetic inference, despite the observation that *CLV2* constitutes a subfamily with *AtRLP30* and *AtRLP38*. Taken together, functional diversity as well as redundancy exists in the *CLV2* subfamily.

The process of subfunctionalization, where the coding regions from closely related family members are still conserved while the cis-regulatory sequences have diverged, is a common phenomenon and has been documented for many gene families (Mazet and Shimeld, 2002; Prince and Pickett, 2002). *CLV1* and the related Barely Any Meristem (BAM1–BAM3) proteins have similar biochemical functions but play opposite roles within the meristem, where BAMs act to promote and *CLV1* to restrict the meristem size. The *BAMs* are broadly expressed in many Arabidopsis organs, which is consistent with their multiple developmental roles (DeYoung et al., 2006), while *CLV1* expression is restricted to the shoot apex (Clark et al., 1997). *CLV1* can fully replace *BAM1* and *BAM2* in developing organs, while *BAM1* and *BAM2* can partially replace *CLV1* function within the meristem when expressed under the *ERECTA* (*ER*) promoter. These results indicate that the distinct functions for these genes are caused by differences in their transcription patterns (DeYoung et al., 2006). Similarly, two *ER* paralogues, *ERL1* and *ERL2*, that display overlapping and distinct expression patterns with the *ER* gene, are capable of substituting the *ER* when expressed under the control of the *ER* promoter (Shpak et al., 2004). While the *er* null allele exhibits compact inflorescences with short lateral organs and internodes (Torii et al., 1996), *erl1* and *erl2* single mutants display no detectable phenotype and

each of them significantly enhances the *er* phenotype (Shpak et al., 2004). Loss of the entire *ER* family conferred many severe defects (Torii et al., 1996; Shpak et al., 2004, 2005; Pillitteri et al., 2007).

To understand which protein domains are essential for *CLV2* function and responsible for its functional specificity, we employed a deletion strategy and exchanged domains between different *AtRLP* proteins. First, we determined which domains are essential for the function of *CLV2*. We generated four deletion constructs, mainly focusing on the C domain, which demonstrated that the *CLV2* island domain (C2) is dispensable for its function. In addition, the results indicate that the C1 and C3 regions are required for proper *CLV2* functioning. The variable C2 region, which splits the variable C1 LRRs and the conserved C3 LRRs, has been suggested to be a flexible hinge-like region forming a loop between the two LRR blocks for folding into the regular LRR structure (Jones and Jones, 1997). A similar island region is also found in particular RLPs and RLKs, for instance Cfs, FEA2, and BRI1 (Jones et al., 1994; Li and Chory, 1997; Thomas et al., 1997; Taguchi-Shiobara et al., 2001), although the island domain does not share any primary sequence similarity between the family members (Torii, 2004). In contrast to our results with *CLV2*, previous studies have highlighted the importance of the island domain of BRI1 in brassinolide perception by RLK BRI1 (Li and Chory, 1997; Friedrichsen et al., 2000; He et al., 2000). Furthermore, the island domain as well as a single LRR motif (LRR22) has been shown to bind directly to brassinolide (Kinoshita et al., 2005), demonstrating the importance of this domain for brassinolide perception. However, the C2 domains of the tomato resistance proteins Cf-4 and Cf-9 are identical, although both proteins recognize different *C. fulvum* avirulence molecules, implying that the C2 domain of these proteins is unlikely to be a determinant for ligand recognition. Consistent with our results, the C2 domains of the functional orthologs FEA2 and *CLV2* are poorly conserved (Jeong et al., 1999; Taguchi-Shiobara et al., 2001), sharing only 15% identity, indicating that this region is not involved in perception of a common ligand. Although in the case of BRI1 the functionality of the C2 domain has been proven, the fact that many functionally characterized RLPs and RLKs lack an island domain, such as Xa21 (Song et al., 1995), *ER* and *ERL1-2* (Torii et al., 1996; Shpak et al., 2004, 2005), *CLV1* (Clark et al., 1997), *FLS2* (Gómez-Gómez and Boller, 2000), *HAESA* (Jinn et al., 2000), *TMM* (Nadeau and Sack, 2002), and *BAM1* to *BAM3* (DeYoung et al., 2006), argues against an important role for this region. Moreover, of the 57 RLPs found in Arabidopsis, 12 lack a C2 island domain (Wang et al., 2008).

Chimeric constructs were designed between *CLV2* and *AtRLP38* to unravel which protein domains determine the functional specificity of *CLV2*. Complementmentation analyses revealed that the C3-G domain of *CLV2* could be replaced by that of *AtRLP38*. The C3-G

region is highly conserved among all RLPs (Fritz-Laylin et al., 2005; Wang et al., 2008) and many functionally characterized RLKs (Jones et al., 1994; Song et al., 1995; Li and Chory, 1997; Gómez-Gómez and Boller, 2000), indicating that this region is required for a generic conserved function either in complex formation or in proper structural folding. In contrast, the complementation tests with the chimeric constructs CLV2RLP38-I, -III, -IV, and -V, in which we replaced a part of or the entire C1 domain of CLV2 by those of AtRLP38, showed that the AtRLP38 C1 region could not substitute the function of the CLV2 C1 domain. The replacement of six C1 LRRs in the CLV2LP38-IV construct already inactivated the function of CLV2. It has been reported that paired Cys residues, located in the C terminus, are probably important residues for RLP and RLK structure and thus functioning (Jeong et al., 1999; Kolade et al., 2006). Although CLV2RLP38-I, -III, -IV, and -V still have these residues, the CLV2 function was lost, indicating that other residues are also important for the function of CLV2. In previous studies, the C1 region has been demonstrated to provide the recognition specificity of Cfs (van der Hoorn et al., 2001, 2005; Wulff et al., 2001; Seear and Dixon, 2003; Rivas et al., 2004). Recently, site-directed mutagenesis of FLS2 also revealed that flagellin perception relies on limited residues in the LRR9 to LRR15 region of FLS2 (Dunning et al., 2007). Furthermore, the C1 domain is highly variable, particularly in the number of LRRs (Shiu and Bleecker, 2001; Fritz-Laylin et al., 2005; Wang et al., 2008). These findings highlight the potential role of the N-terminal LRRs of the RLPs and RLKs in the determination of functional specificity. Furthermore, polymorphisms of the CLV2 gene in different ecotypes suggest that LRR1 to LRR4 may functionally not be as important as the remaining parts of the protein (Jeong et al., 1999). This observation, as well as our domain-deletion and domain-swap results, strongly suggests that LRR5 to LRR17 of CLV2 play a critical role either in the ligand-binding specificity or in dimerization with partner proteins that are required for the CLV signaling pathway. Taken together, our studies provide valuable information on the functions of CLV2 domains and how these domains are conserved among related AtRLP family members.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* ecotypes Col-0 and *Ler* were used as wild types. The *atrlp10* (*clv2-7*), *atrlp2*, *atrlp12*, and *clv2-3* mutants were described previously (Kayes and Clark, 1998; Wang et al., 2008). The genotypes of the double mutants were determined by PCR using gene-specific primer pairs and a combination of T-DNA and gene-specific primers as described previously (Wang et al., 2008). Seeds were gas sterilized for 1 h by mixing 100 mL of bleach (containing 4% NaClO) and 3 mL of HCl. Subsequently, the seeds were germinated on half-strength Murashige and Skoog medium containing 1% Suc, 0.5 g/L MES, and 0.8% (w/v) agar at pH 5.8. Plants were grown on soil at 22°C either under short-day (8-h-day/16-h-night) or long-day (16-h-day/8-h-night) conditions.

Complementation of the *clv2* Mutant by CLV2 Homologs

Full-length CLV2 ($P_{CLV2}:CLV2$) including the native promoter and terminator was amplified using the primers GD1 and GD2 including the Gateway cloning sites. PCR was performed using Col-0 genomic DNA as template. The CLV2 promoter and *AtRLP* gene fragments for different constructs were generated using GD1/GD3 and GD4/GD5 for $P_{CLV2}:AtRLP2$, GD1/GD6 and GD7/GD8 for $P_{CLV2}:AtRLP12$, GD1/GD9 and GD10/GD11 for $P_{CLV2}:AtRLP30$, GD1/GD12 and GD13/GD14 for $P_{CLV2}:AtRLP38$, GD1/GD15 and GD16/GD17 for $P_{CLV2}:TMM$, and GD1/GD18 and GD19/GD20 for $P_{CLV2}:Cf-9$ (Supplemental Table S1). The P_{CLV2} and PCR fragments were combined using overlap extension PCR, after which the complete constructs were generated via PCR using primers GD1 and GD5 for $P_{CLV2}:AtRLP2$, GD1 and GD8 for $P_{CLV2}:AtRLP12$, GD1 and GD11 for $P_{CLV2}:AtRLP30$, GD1 and GD14 for $P_{CLV2}:AtRLP38$, GD1 and GD17 for $P_{CLV2}:TMM$, and GD1 and GD20 for $P_{CLV2}:Cf-9$ (Supplemental Table S1). The PCR fragments were recombined into *pDONR207* (Invitrogen) by a BP reaction, and subsequently all fragments were cloned into the binary vector with an LR reaction using the plasmids from the BP reaction mixed with the *pKGW* vector (Karimi et al., 2002) following the supplier's protocol. The plasmids were introduced into *Agrobacterium tumefaciens* strain C58mp90 by electroporation and transformed into the *clv2-3* mutant plants by floral dip transformation (Clough and Bent, 1998).

Generation of Deletion and Chimeric Constructs and Transformation

As for the deletion constructs, the two fragments of the deletion constructs ($\Delta 1$ – $\Delta 4$) were generated using GD1/GD21 and GD2/GD22 for construct $\Delta 1$, GD1/GD23 and GD2/GD24 for construct $\Delta 2$, GD1/GD25 and GD2/GD26 for construct $\Delta 3$, and GD1/GD27 and GD2/GD28 for construct $\Delta 4$ (Supplemental Table S1). The two fragments were combined with overlap extension PCR, after which the four deletion constructs were generated via PCR using primers GD1 and GD2 (Supplemental Table S1). For the chimeric constructs between CLV2 and *AtRLP38*, the fragments for each construct were amplified using GD1/GD29 and GD2/GD30 for $P_{CLV2}:CLV2RLP38-I$, GD1/GD31 and GD32/GD33 for $P_{CLV2}:CLV2RLP38-II$, GD1/GD34 and GD33/GD35 for $P_{CLV2}:CLV2RLP38-III$, GD1/GD36 and GD33/GD37 for $P_{CLV2}:CLV2RLP38-IV$, and GD1/GD38 and GD2/GD39 for $P_{CLV2}:CLV2RLP38-V$ (Supplemental Table S1). Subsequently, the two fragments were combined with overlap extension PCR by primers GD1 and GD2 for $P_{CLV2}:CLV2RLP38-I$ and $P_{CLV2}:CLV2RLP38-V$ and primers GD1 and GD33 for $P_{CLV2}:CLV2RLP38-II$, $P_{CLV2}:CLV2RLP38-III$, and $P_{CLV2}:CLV2RLP38-IV$ (Supplemental Table S1). All resulting PCR fragments were recombined into *pDONR207* and subsequently cloned into the *pKGW* vector (Karimi et al., 2002). The binary vectors, containing the different fragments, were sequenced before transformation to the *clv2-3* mutants. Transgenic plants were created by floral dip transformation (Clough and Bent, 1998).

RNA Isolation and Reverse Transcription-PCR

Total RNA was isolated from inflorescences using the Qiagen RNeasy Kit according to the manufacturer's instructions. Residual genomic DNA contamination was removed with RNase-free DNase I and further purified. First-strand cDNA was synthesized from 1 μ g of DNA-free RNA with Moloney murine leukemia virus reverse transcriptase at 37°C for 50 min. The primers used for $\Delta 1$, $\Delta 2$, and $\Delta 3$ are GD40 and GD41, while GD41 and GD42 were used for $\Delta 4$ (Supplemental Table S1). The primer pairs GD43 and GD44 (Supplemental Table S1) were used to amplify the *ACTIN* gene as a control.

Supplemental Data

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

Supplemental Figure S1. Domain organization of CLV2-like AtRLPs.

Supplemental Figure S2. Comparison of expression profiles of CLV2 subfamily genes.

Supplemental Figure S3. Several CLV2-like genes display an expression in SAM.

Supplemental Figure S4. Expression of deletion constructs.

Supplemental Figure S5. Sequence alignment of full-length CLV2 and AtRLP38 proteins.

Supplemental Figure S6. Domain composition comparison between CLV2 and AtRLP38 proteins.

Supplemental Table S1. Primer sequences used in this study.

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