The Arabidopsis CLAVATA2 Gene Encodes a Receptor-like Protein Required for the Stability of the CLAVATA1 Receptor-like Kinase

Sangho Jeong, Amy E. Trotochaud, and Steven E. Clark
Department of Biology, University of Michigan, Ann Arbor, Michigan 48109-1048

The CLAVATA2 (CLV2) gene regulates both meristem and organ development in Arabidopsis. We isolated the CLV2 gene and found that it encodes a receptor-like protein (RLP), with a presumed extracellular domain composed of leucine-rich repeats similar to those found in plant and animal receptors, but with a very short predicted cytoplasmic tail. RLPs lacking cytoplasmic signaling domains have not been previously shown to regulate development in plants. Our prior work has demonstrated that the CLV1 receptor-like kinase (RLK) is present as a disulfide-linked multimer in vivo. We report that CLV2 is required for the normal accumulation of CLV1 protein and its assembly into protein complexes, indicating that CLV2 may form a heterodimer with CLV1 to transduce extracellular signals. Sequence analysis suggests that the charged residue in the predicted transmembrane domain of CLV2 may be a common feature of plant RLPs and RLKs. In addition, the chromosomal region in which CLV2 is located contains an extremely high rate of polymorphism, with 50 nucleotide and 15 amino acid differences between Landsberg erecta and Columbia ecotypes within the CLV2 coding sequence.

INTRODUCTION

The shoot meristem is responsible for the formation of all above-ground organs in higher plants. This task is accomplished by balancing two processes: the maintenance of a population of undifferentiated cells at the center of the shoot meristem and the direction of progeny cells on the flanks of the meristem toward organ formation and eventual differentiation. Mutations in several genes in Arabidopsis have been shown to disrupt this balance (reviewed in Clark, 1997), including SHOOT MERISTEMLESS (STM), WUSCHEL (WUS), and the genes comprising the CLAVATA loci (CLV1, CLV2, and CLV3) (Leyser and Furner, 1992; Barton and Poethig, 1993; Clark et al., 1993, 1995; Laux et al., 1996; Hayes and Clark, 1998). Plants carrying mutations at the CLV loci accumulate undifferentiated cells in the shoot and flower meristems, and it has therefore been proposed that the CLV genes either inhibit the division of these cells or promote their transition toward a differentiated state (reviewed in Clark, 1997).

The phenotypes of clv1 and clv3 mutants are identical, and these two genes function in the same pathway to regulate meristem development. In addition, the phenotypes of the clv1 and clv3 mutants and the gene expression patterns for CLV1 and CLV3 appear to be specific to the shoot and flower meristems (Clark et al., 1993, 1995, 1997; Fletcher et al., 1999). Compared with clv1 and clv3 mutants, all clv2 mutant alleles display weak phenotypes at the shoot and flower meristems (Kayes and Clark, 1998). clv2 mutations also affect the development of several organ types. These include the pedicel, which is elongated in clv2 mutants, and the anthers and valves of the flower, both of which are reduced in clv2 mutants. Genetic analysis indicates that CLV2 functions in the same pathway as CLV1 and CLV3 in the regulation of meristem development but in a separate pathway in the regulation of organ development (Kayes and Clark, 1998).

CLV1 and CLV3 appear to encode components of a signal transduction pathway. The predicted CLV1 protein is a receptor-like kinase (RLK), with extracellular leucine-rich repeats (LRRs), a single transmembrane domain, and a functional intracellular serine protein kinase domain (Clark et al., 1997; Williams et al., 1997; Stone et al., 1998). CLV3 encodes a potential ligand in the form of a small, secreted protein (Fletcher et al., 1999). Evidence for the role of CLV3 in the activation of CLV1 has come from analysis of CLV1 protein complexes in vivo (Trotochaud et al., 1999). CLV1 is found in two protein complexes in cauliflower and Arabidopsis. One complex is an inactive disulfide-linked multimer of 185 kD, and the other, which is presumed to be the active complex, is a 450-kD complex that includes the 185-kD
CLV1 multimer as well as additional proteins. Inferences as to the function of these two complexes (active and inactive) are based on analysis of CLV1 complex formation in mutant plants. In a clv1 mutant with a partial loss of in vitro kinase activity (clv1-1), a reduction of the proportion of CLV1 in the 450-kD complex was observed, whereas in a clv1 mutant with a complete loss of in vitro kinase activity (clv1-10), a complete loss of the 450-kD complex was observed. In clv3 mutants, a complete loss of the 450-kD complex was also observed, indicating that CLV3 is required for CLV1 activation (Trotochaud et al., 1999).

Additional components of the 450-kD complex have been identified (Trotochaud et al., 1999). One component is the kinase-associated protein phosphatase (KAPP), which was shown previously to interact with several protein kinases (Stone et al., 1994; Braun et al., 1997) and to be a negative regulator of CLV1 signal transduction (Williams et al., 1997; Stone et al., 1998). The 450-kD complex also contains a Rho GTPase-related protein that presumably relays signals to downstream components (Trotochaud et al., 1999).

Because CLV2 functions in the same pathway as CLV1 and CLV3 to regulate meristem development, we considered it a reasonable hypothesis that CLV2 may code for an additional component of one or both of the CLV1 complexes. However, the CLV2 protein must be unique in function because inactivating CLV2 results in a weaker phenotype than does inactivating CLV1 or CLV3 and because CLV2 regulates the development of different organ types independently of CLV1 and CLV3 (Kayes and Clark, 1998). Thus, we undertook to isolate the CLV2 gene. We report here that it encodes a receptor-like protein (RLP) predicted extracellular LRRs but only a very short cytoplasmic domain. Consistent with a role in CLV1 protein function, we demonstrate that CLV2 is required for CLV1 protein accumulation. Possible roles for CLV2 are as a heterodimer partner for CLV1 to detect extracellular signals within the shoot meristem and as a heterodimer partner with other RLKs to regulate the development of organs such as the pedicel and gynoecium.

RESULTS

CLV2 Cloning

The clv2-5 mutant allele was isolated from T-DNA insertion mutagenized populations (Feldmann, 1991; Kayes and Clark, 1998). By outcrossing to wild-type Arabidopsis, we observed tight linkage between the clv2 phenotype and kanamycin resistance (data not shown). The clv2-5 genomic region surrounding the T-DNA insertion was cloned and sequenced. A complex, highly rearranged T-DNA insertion was found to have disrupted the open reading frame (ORF) of a gene encoding an RLP (Figure 1). Sequences for this

Figure 1. CLV2 Encodes an RLP.

(A) The genomic organization at the CLV2 locus, indicating the site of T-DNA insertion in the clv2-5 allele. The original T-DNA structure is shown at the top, with its domains color coded. The best estimate of the T-DNA structure after insertion is shown in the middle. The orientations of the colored arrows represent the orientations of the corresponding domains after insertion. Restriction sites used for sequencing are indicated (E, EcoRI; H, HindIII; S, SacI). The region from the wild-type Col ecotype genome used to complement clv2 is also shown. LB, left border; Amp', ampicillin resistance; Tn903, transposon 903; Kan', kanamycin resistance; Tet', tetracyycin resistance; RB, right border. (B) to (J) Regions of the predicted CLV2 protein, separated into the signal peptide (B), the cysteine-pair regions (C) and (G), the LRRs (D) and (F), the “island” (E), the acidic domain (H), the transmembrane domain (I), and the cytoplasmic stop transfer sequence (J). Red amino acids indicate residues that are polymorphic among different ecotypes. Dashes indicate gaps introduced into the sequence to maximize alignment. The GenBank accession numbers for the CLV2 gene and protein sequence from Col, Ler, and Ws are AF177672, AF177673, and AF177674, respectively.
CLV2 Encodes a Receptor-like Protein

Table 1. clv2 Alleles

<table>
<thead>
<tr>
<th>Allele</th>
<th>Lesion</th>
<th>Predicted Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>clv2-1</td>
<td>C→T at 97</td>
<td>Gln-33→stop codon</td>
</tr>
<tr>
<td>clv2-2</td>
<td>1-bp deletion at 1281</td>
<td>Frameshift</td>
</tr>
<tr>
<td>clv2-3</td>
<td>61-bp deletion from 1095 to 1155</td>
<td>Frameshift</td>
</tr>
<tr>
<td>clv2-4</td>
<td>C→T at 97</td>
<td>Gln-33→stop codon</td>
</tr>
<tr>
<td>clv2-5</td>
<td>T-DNA insertion at 2158</td>
<td>Frameshift</td>
</tr>
</tbody>
</table>

* Indicates that nucleotides are numbered from the predicted translation start site.

ORF were compared among all five clv2 alleles and their corresponding wild-type ecotypes. This revealed lesions leading to premature termination codons in all mutant alleles, suggesting that this ORF corresponded to the CLV2 gene (Table 1) and that the clv2 mutations are likely null alleles. To confirm that this ORF was CLV2, we transformed a 4.9-kb fragment from the wild-type genomic region into clv2-3 mutants (Figure 1A). Of 47 primary transformants, 44 exhibited a complete suppression of the mutant phenotype (data not shown).

Predicted CLV2 Protein Structure

The CLV2 gene appears to contain a single exon that codes for a 720-amino acid protein with a characteristic signal peptide (Figures 1B to 1I). 5' Rapid amplification of cDNA ends (RACE) analysis indicated a single major transcription initiation site 153 bp upstream of the predicted start ATG (see Methods). An expressed sequence tag (GenBank accession numbers AA586072 and T41629) for this gene contains the predicted stop codon, 3' untranslated region, and polyadenylation tail, indicating that no unidentified exons exist downstream.

The majority of the predicted CLV2 protein consists of LRRs. The alignment for these repeats is shown in Figures 1D and 1F. The LRRs are flanked by cysteine pairs that are each separated by seven residues, a motif common to extracellular LRR proteins (Jones and Jones, 1996) (Figures 1C and 1G). The CLV2 protein shares similarity with a growing family of receptors and RLKs found in both plants and animals (Jiang et al., 1995; Lease et al., 1998). However, the CLV2 protein lacks a kinase domain. Receptor-like LRR proteins without any cytoplasmic signaling domain are relatively rare, and only one family has been previously described in plants. This is the Cf family of genes from tomato, which provides resistance to specific races of the fungus Cladosporium fulvum (Jiang et al., 1994; Dixon et al., 1996). Both the CLV2 and the Cf RLPS have a very short predicted cytoplasmic domain and an island of non-LRR sequence between repeats 4 and 5, counting from the transmembrane domain (Figures 1E and 1J). CLV2 and Cf proteins also share an acidic domain immediately adjacent to the transmembrane domain (Figure 1H).

CLV2 Is Expressed in Many Tissues

Because CLV2 is required for the proper development of many organ types, we predicted that CLV2 should be expressed in many different tissues. Total RNA was collected from apices (shoot meristems and unopened flower meristems), opened flowers, siliques, 4-day-old seedlings, and rosette leaves and used for RNA gel blot analysis. Whereas the highest expression was detected in apices, as expected, CLV2 mRNA was detected in all tissues (Figure 2A).

To determine whether the weaker signal detected in some of the organ types was the result of hybridization to a related sequence, we tested the Arabidopsis genome for cross-hybridizing sequences. Low-stringency DNA gel blot hybridization using the entire CLV2 gene as a probe detected only the CLV2 genomic sequence (Figure 2B), indicating that no closely related genes exist in Arabidopsis. Moreover, no highly similar DNA sequences were identified by BLAST analysis (Altschul et al., 1997).

Figure 2. CLV2 Is Expressed in All Tissues Tested.

(A) Twenty micrograms of total RNA isolated from wild-type Arabidopsis 4-day-old seedlings (lane 1), rosette leaves (lane 2), siliques (lane 3), opened flowers (lane 4), and apices (shoot meristems and unopened flowers; lane 5) was used to prepare an RNA gel blot that was probed consecutively with a portion of the CLV2 coding region and an 18S rDNA control.

(B) Genomic DNA from wild-type Col (lane 1), Ler (lane 2), and Ws (lane 3) ecotypes was digested with EcoRI and used to generate a DNA gel blot probed with the entire CLV2 coding region. This was washed at low stringency. Note that the Ws genome has a polymorphic EcoRI site at position 433 in the coding region, resulting in two hybridizing bands.
CLV2 Is Required for CLV1 Protein Accumulation

Because CLV2 functions in the same pathway as CLV1 to regulate meristem development (Kayes and Clark, 1998), we hypothesized that the CLV2 gene product could interact with the CLV1 RLK. We have previously demonstrated that CLV1 is found in two distinct protein complexes in vivo: a disulfide-linked 185-kD complex and a larger 450-kD complex that includes the 185-kD multimer, the protein phosphatase KAPP, and a Rho GTPase–related protein (Trotochaud et al., 1999). We have proposed that the 185-kD complex is the inactive form of CLV1 and may represent a CLV1 heterodimer with a second RLK. That the 450-kD CLV1 complex is absent in clv1 and clv3 mutant backgrounds and is associated with a Rho-related signaling protein suggest that the 450-kD complex contains the active form of CLV1. If CLV2 is a component of either complex, then eliminating the CLV2 protein should alter complex size and/or accumulation.

To test this hypothesis, we first examined CLV1 protein levels in wild-type and clv2-3 plants by protein gel blotting, as shown in Figure 3A. We did not detect cross-reaction of the CLV1 antiserum to extracts from clv2-3 plants, suggesting a significant reduction in CLV1 protein in these plants. To quantify this difference precisely, we performed ELISA assays on extracts from wild-type, clv2-2, and clv2-3 plants, revealing a >90% reduction in CLV1 accumulation in clv2 plants (Figure 3B). In a control procedure, the same extracts from clv2 plants were shown to have a normal amount of KAPP protein (Figure 3B). Because we have previously shown that clv2 mutants have normal, if not higher, levels of CLV1 transcription (Kayes and Clark, 1998), any changes in CLV1 protein accumulation must be due to post-transcriptional mechanisms. ELISA analysis of gel chromatography fractions revealed that the CLV1 protein that is present in clv2 mutants is primarily found in a novel, higher molecular mass complex of ~600 kD (Figure 3C).

Unprecedented Polymorphisms

While determining the sequences of the clv2 mutant alleles, we observed a very high level of sequence polymorphism between various Arabidopsis ecotypes. We observed 50 nucleotide polymorphisms between Landsberg erecta (Ler) and Columbia (Col) and 54 between Ler and Wassilewskija (Ws) (Table 2). In total, 68 sites were found to be polymorphic. These polymorphisms have a significant effect on the predicted amino acid sequences of the CLV2 protein from the various ecotypes. Red residues in Figure 1 indicate those that are polymorphic between the ecotypes. Most are clustered in a short region of sequence in the N-terminal portion of the predicted protein. The changes predominantly alter those residues that are not part of the LRR consensus. The three consensus residues that are polymorphic are subjected to conservative substitutions: residue 484 is valine (Ler and Col) and isoleucine (Ws); residue 532 is phenylalanine (Col), leucine (Ler), and isoleucine (Ws); and residue 739 is valine (Ler) and isoleucine (Col and Ws).

To determine whether the polymorphisms might reflect selection for modified forms of CLV2, we examined the amount of sequence polymorphism in the coding sequences of the adjacent serpin ORF and in the intergenic region between the CLV2 and serpin ORFs. These regions also contained a very high degree of sequence polymorphism. The serpin ORF contained 20 sites that were polymorphic between the Ler, Col, and Ws ecotypes over the 1068 bp of the predicted coding sequences. These polymorphisms included two deletions/insertions that are predicted to disrupt
The identification of the CLV2 gene revealed a predicted RLP that represents a novel class of developmental regulators. CLV2 is the first RLP lacking a cytoplasmic signaling domain that has been shown to regulate development in plants. Previously, the only known functional RLPS were those encoded by the Cf gene family from tomato, which is required for recognition of specific races of the fungus C. fulvum (Jones et al., 1994; Dixon et al., 1996).

CLV2 Function

How does the identification of the CLV2 gene sequence help us to understand its role in development? CLV2 operates in the same pathway as CLV1 and CLV3, whose gene products appear to function as components of a signal transduction cascade. CLV1 encodes an RLK (Clark et al., 1997), whereas CLV3 encodes a small, secreted protein (Fletcher et al., 1999). CLV3 is also required for the activation of the CLV1 protein in vivo (Trotochaud et al., 1999). Because CLV2 functions in the same pathway as CLV1 and CLV3, we would expect CLV2 to function as a component of this signal transduction cascade. Based on the predicted CLV2 protein structure, one possible role for CLV2 in CLV1/CLV3 signaling could be to act as a heterodimer partner with CLV1. This would be consistent with the size of the inactive complex, which is ~185 kD. However, size determination by column chromatography can be influenced by several variables, and the actual mass of the components of this complex may add up to significantly more or less than 185 kD. Thus, although the size of the inactive CLV1 complex is consistent with a CLV1/CLV2 heterodimer, it does not rule out other possibilities, such as a CLV1 homodimer.

Additional evidence for CLV1/CLV2 heterodimers came from analysis of CLV1 protein amounts in the clv2 mutant background. We observed that >90% of the CLV1 protein was absent in these plants, despite the fact that we have previously shown that CLV1 is transcribed at normal, if not higher, levels in clv2 plants (Kayes and Clark, 1998). Thus, the loss of CLV1 protein in clv2 mutants reflects post-transcriptional regulation. In many other systems, when one member of a heterodimer pair is absent, the partner protein is degraded in the endoplasmic reticulum (Hurtley and Helenius, 1989). The CLV2 secreted protein, is consistent with a role for CLV3 as the ligand for CLV1 (Fletcher et al., 1999). If this remaining CLV1 protein is active, then it would explain why all clv2 mutant alleles are weak in phenotype compared with clv3 mutants.

**DISCUSSION**

The identification of the CLV2 gene revealed a predicted RLP that represents a novel class of developmental regulators. CLV2 is the first RLP lacking a cytoplasmic signaling domain that has been shown to regulate development in plants. Previously, the only known functional RLPS were those encoded by the Cf gene family from tomato, which is required for recognition of specific races of the fungus C. fulvum (Jones et al., 1994; Dixon et al., 1996).
If CLV1 forms a heterodimer with CLV2, then binding would likely be mediated by disulfide linkage between conserved cysteine pairs flanking the LRR regions. We have previously shown that all CLV1 protein in vivo is in a disulfide-linked multimer of 185 kD (Trotchoud et al., 1999). Charge interaction in the extracellular sequences immediately adjacent to the putative transmembrane domain could also play a role in heterodimer formation. In this region, CLV1 is basic, whereas CLV2 is acidic. This type of charge interaction in an extracytoplasmic peritransmembrane (EPTM) domain was suggested as a mechanism for major histocompatibility complex II (β2) heterodimer formation (Cosson and Bonifacino, 1992). Because CLV2 functions in a pathway separate from CLV1 in the regulation of organ development, one possibility is that CLV2 forms a heterodimer with a separate RLK in these tissues.

How LRR-containing RLPS that lack a cytoplasmic domain can transmit signals across the plasma membrane is unknown. Heterodimer formation between CLV1 and CLV2 provides a possible mechanism by which this class of RLPS may function as signaling components. That is, these RLPS may form heterodimers with an RLK to form a functional complex with both a receptor domain and a cytoplasmic signaling domain. Interestingly, Xa21, a rice RLK that provides resistance to a specific race of Xanthomonous spp, was shown to function without a transmembrane or cytoplasmic domain (Wang et al., 1998). Perhaps the truncated form of Xa21 functions in a manner analogous to CLV2.

### Transmembrane Charge

An interesting aspect of the deduced CLV2 amino acid sequence is the presence of an aspartic acid residue in the predicted transmembrane domain. There are only a few examples of single-pass transmembrane RLPS containing a charged residue in the transmembrane domain (Harrison, 1996). The best-studied example is the T-cell receptor (TCR) complex. The components of the TCR complex contain charged residues in their transmembrane domains that have been shown to be critical for protein-protein interactions and complex formation (Cosson et al., 1991).

To address the potential significance of the charged residues in the transmembrane and EPTM domains in plant RLPS and RLK function, we analyzed 54 additional RLPS and RLKs identified from the Arabidopsis genome database. Of the sequences analyzed, 43 encoded apparent RLPS, whereas 12 (including CLV2) encoded RLPS (Table 3). Six of the RLPS and seven of the RLPS contained charged residues in their predicted transmembrane domain. These were evenly split between basic and acidic residues, with most of the RLPS containing acidic residues and most of the RLPS containing basic residues. Thus, charged transmembrane domain residues appear to be a common feature of plant RLPS and RLKs. Our analysis also indicated that all RLPS have basic EPTM domains and that all RLPS have acidic EPTM domains (Table 3). The opposite charges of the EPTM residues may mediate heterodimer formation between RLPS and RLKs. Although CLV1 lacks a corresponding charged residue in its predicted transmembrane domain, the charged amino acid in CLV2 could mediate interaction with an RLK in its regulation of organ development or could mediate interaction with other components of the CLV1 signaling complex.

#### CLV2 Polymorphisms

The region of chromosome 1 in which CLV2 is located has the highest level of polymorphism reported to date for single-copy gene regions of Arabidopsis. The only other reports of highly polymorphic regions in Arabidopsis are the gene clusters of disease resistance loci (Botella et al., 1998; McDowell et al., 1998). The 68 polymorphic sites over 2163 bp represented at least an order of magnitude increase over that typically found among the Col, Ler, and Ws ecotypes for other genes. For example, the CLV1 gene, also located on chromosome 1, has no polymorphic sites between the Ler and Col ecotype over 2940 bp of coding sequence. The ratio of synonymous to nonsynonymous changes (2.4:1) and the proportion of third-position to first- and second-position changes (2:1) found between Ler and Ws suggest that CLV2 remains under selection (Table 4). Indeed, despite the amino acid polymorphisms, the CLV2 alleles found in each of the three ecotypes are functionally similar, based on the similarity of their phenotypes when mutated (Kayes and Clark, 1998).

This high amount of polymorphism likely does not reflect selection for altered versions of CLV2. This conclusion is based on the observation that the adjacent gene and the

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**Table 3. Charged Residues in Predicted Transmembrane Domains of RLPS and RLKs**

<table>
<thead>
<tr>
<th>Type</th>
<th>Total</th>
<th>No Charge</th>
<th>His</th>
<th>Asp</th>
<th>Lys</th>
<th>Basic</th>
<th>Acidic</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLK</td>
<td>43</td>
<td>37</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>43</td>
<td>0</td>
</tr>
<tr>
<td>RLP</td>
<td>12</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
</tbody>
</table>

a The predicted CLV2 amino acid sequence was used in a BLAST analysis (Altschul et al., 1997) to identify fully sequenced homologous genes (partial clones and expressed sequence tags were not included).

b The number of predicted gene products in each category containing no charged residues (No charge), histidine (His), aspartic acid (Asp), and lysine (Lys) in their putative transmembrane domains.

The first 10 residues from the predicted transmembrane domain were assessed to determine if the putative EPTMs were basic or acidic overall.
Table 4. Summary of Polymorphisms in the CLV2 Gene

<table>
<thead>
<tr>
<th>Ecotypes</th>
<th>Totala</th>
<th>Tsb</th>
<th>Tsc</th>
<th>1stc</th>
<th>2nd</th>
<th>3rd</th>
<th>Syned</th>
<th>Nsyf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col vs. Ler</td>
<td>50</td>
<td>41</td>
<td>9</td>
<td>13</td>
<td>3</td>
<td>34</td>
<td>35</td>
<td>15</td>
</tr>
<tr>
<td>Col vs. Ws</td>
<td>34</td>
<td>23</td>
<td>11</td>
<td>7</td>
<td>2</td>
<td>25</td>
<td>27</td>
<td>7</td>
</tr>
<tr>
<td>Ler vs. Ws</td>
<td>54</td>
<td>40</td>
<td>14</td>
<td>13</td>
<td>5</td>
<td>36</td>
<td>38</td>
<td>16</td>
</tr>
</tbody>
</table>

aTotal number of polymorphic sites.
bTransitions.
cTransversions.
dPolymorphisms at the first, second, and third codon positions.
eSynonymous changes.
fNon-synonymous changes.

INTERGENIC REGION

The intergenic region contain similar levels of polymorphism. Thus, the changes in the CLV2 sequence indicate that this region of chromosome 1 is subjected to a very high level of mutagenesis. Why this region is mutagenic is unclear. It is unlikely due to high levels of methylation, because the ratio of transitions to transversions (2.9:1 between Ler and Ws) is not unusually high. Other genes that have been shown to have high levels of polymorphism in Arabidopsis (although not as high as CLV2) include AP3 (APETALAE3; Purugganan and Suddith, 1999), RPS2 (RESISTANCE TO PSEUDOMONAS SYRINGAE2; Caicedo et al., 1999), and CAL (CAULIFLOWER; Purugganan and Suddith, 1998). Interestingly, the highly rearranged T-DNA insertion in the clv2-5 line (Figure 1A) may reflect the genomic instability in this region.

If the high level of polymorphism is consistent in other ecotypes, then sequencing of CLV2 in additional ecotypes may provide a wealth of structure-function information. The polymorphisms detected in the Ler, Col, and Ws ecotypes suggest that LRR repeats 1 to 4 may not be functionally as important as the remaining portion of the protein, because the majority of first- and second-position substitutions occur in this region.

METHODS

CLV2 Cloning

A λ genomic library was generated using DNA isolated from clv2-5 homozygous Arabidopsis thaliana plants and was screened by using the neomycin phosphotransferase nptII sequence from the T-DNA as a probe. Five hybridizing λ clones were isolated, subcloned, and partially sequenced. Plant DNA on each side of the T-DNA insertion was used to probe a λ genomic library made from wild-type Columbia (Col). Two λ clones hybridizing to both flanking probes were isolated and analyzed. The single expressed sequence tag for the CLAVATA2 gene (GenBank accession numbers AA86072 and T41629) was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH).

DNA Gel Blot Analysis

The entire CLV2 gene was used as a probe. Hybridization was conducted overnight at 65°C in 0.25 M sodium phosphate, pH 7.4, 1 mM EDTA, 1% casein, and 7% SDS. The filter was washed twice with 2 × SSPE (1 × SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 10 mM EDTA, pH 7.4) and 0.1% SDS at room temperature for 10 min each and then with 0.5 × SSPE and 0.1% SDS at 50°C for 20 min.

Rapid Amplification of cDNA Ends

For 5′ rapid amplification of cDNA ends (RACE), total RNA was isolated from shoot apices using the RNeasy plant mini kit (Qiagen, Chatsworth, CA), and mRNA was purified using the PolyATtract mRNA isolation system (Promega). 5′ RACE PCR was performed with a 5′ RACE kit (Boehringer Mannheim) according to the manufacturer’s instructions. Primers used for reverse transcription and PCR reactions were as follows: primer 1, 5′-GCTAAAACCTGTCCAGTTAGAA-3′; primer 2, 5′-TCAGATCGAAAGCTGAGAT-3′; and primer 3, 5′-TCAACGGGAAGAGATAGTT-3′. A major PCR product was gel-purified and sequenced by direct PCR sequencing with an ABI330 genetic analyzer.

RNA Gel Blot Analysis

Plant tissues were collected from shoot apices with unopened flowers, opened flowers, rosette leaves, green siliques, and 4-day-old seedlings and were immediately frozen in liquid nitrogen. Total RNAs were extracted from each tissue using the RNeasy plant mini kit. Twenty micrograms of total RNA was used for blotting. The NorthernMax kit (Ambion, Austin, TX) was used for RNA gel blot hybridization, according to the manufacturer’s instructions. A 945-bp fragment was amplified using primers 5′-TGGATCCAGAGTCGAGACGACGAGTGTGTGAT-3′ from the CLV2 coding region and cloned into the pCR2.1 vector (Stratagene, La Jolla, CA) to generate an RNA probe with the riboprobe in vitro transcription system (Promega).
Plant Growth Conditions

Seeds were sown on a 1:1:1 mix of top soil:perlite:vermiculite and imbibed for 5 days at 4°C. Plants were grown at 20°C under 900 foot-candles of constant cool white fluorescent light and watered with 0.05 g/L 20-20-20 fertilizer (Knox Fertilizer Co., Knox, IN). Seedlings used for RNA analysis were grown on plates containing Murashige and Skoog salts (Murashige and Skoog, 1962), pH 5.7, 0.8% agar, and 1% sucrose.

Plant Transformation

The 4.9-kb genomic fragment was subcloned into binary vector pPZP 222 (Hajdukiewicz et al., 1994) and transformed into Agrobacterium tumefaciens GV3101. Wild-type Ler and clv2-3 plants were transformed by the vacuum infiltration method (Bechtold et al., 1993). Transformants were selected on plates with gentamycin, and segregation of clv2-3 plants in the next generation was observed.

Protein Analysis

All protein work was performed as described by Trotochaud et al. (1997). The 4.9-kb genomic fragment was subcloned into binary vector pPZP 222 (Hajdukiewicz et al., 1994) and transformed into Agrobacterium tumefaciens GV3101. Wild-type Ler and clv2-3 plants were transformed by the vacuum infiltration method (Bechtold et al., 1993). Transformants were selected on plates with gentamycin, and segregation of clv2-3 plants in the next generation was observed.

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Sangho Jeong, Amy E. Trotochaud and Steven E. Clark
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