Role of the ACL2 locus in flower stalk elongation in Arabidopsis thaliana

Hiroaki Kato¹, Yoshibumi Komeda², Tamao Saito³, Hidetaka Ito⁴,⁵ and Atsushi Kato⁴*

¹Biosystems Science Course, Graduate School of Life Science, Hokkaido University, Kita10 Nishi8, Kita-ku, Sapporo, Hokkaido 060-0810, Japan
²Department of Biological Sciences, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
³Department of Materials and Life Sciences, Faculty of Science and Technology, Sophia University, 7-1 Kioicho, Chiyoda-ku, Tokyo 120-8554, Japan
⁴Department of Biological Sciences, Faculty of Science, Hokkaido University, Kita10 Nishi8, Kita-ku, Sapporo, Hokkaido 060-0810, Japan
⁵Japan Science and Technology Agency, PRESTO, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

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The acaulis2 (acl2) mutant of Arabidopsis thaliana shows a defect in flower stalk elongation. We identified the mutation point of acl2 by map-based cloning. The ACL2 locus is located within an approximately 320-kb region at around 100 map units on chromosome 1. One nucleotide substitution was detected in this region in the acl2 mutant, but no significant open reading frames were found around this mutation point. When wild-type DNA fragments containing the mutation point were introduced into acl2 mutant plants, some transgenic plants partially or almost completely recovered from the defect in flower stalk elongation. 3'-RACE experiments showed that bidirectional transcripts containing the acl2 mutation point were expressed, and the Plant MPSS database revealed that several small RNAs were produced from this region. Microarray analysis showed that transcription of many genes is activated in flower stalks of acl2 mutant plants. Overexpression of some of these genes caused a dwarf phenotype in wild-type plants. These results suggest the following novel mechanism for control of the elongation of flower stalks. Bidirectional non-coding RNAs are transcribed from the ACL2 locus, and small RNAs are generated from them in flower stalks. These small RNAs repress the transcription of a set of genes whose expression represses flower stalk elongation, and flower stalks are therefore fully elongated.

Key words: acaulis2 mutation, Arabidopsis thaliana, flower stalk, non-coding RNA, small RNA

INTRODUCTION

Plant development is divided to two phases, the vegetative phase and the reproductive phase. In the rosette-type plant Arabidopsis thaliana, the plant forms rosette leaves and elongation of flower stalks does not occur during the vegetative phase; flower buds are produced and flower stalks start to elongate after the transition to the reproductive phase. The bolting of flower stalks is thought to be tightly coupled to the flowering process and is strictly regulated by genetic factors in Arabidopsis.

Flower stalk elongation is an important event for reproductive strategies in plants. To study the mechanisms involved in the development of flower stalks, several mutants that are defective in elongation of flower stalks have been isolated and named aculis (acl) (Tsukaya et al., 1993).

Dwarf mutants have been isolated in many studies. Some of them are related to phytohormone deficiency. Gibberellins (GAs) participate in plant development processes including stem elongation, and some dwarf mutants are GA-insensitive or defective in GA biosynthesis (Hedden and Kamiya, 1997; Claey's et al., 2014). Brassinosteroid also controls plant growth, and loss of brassinosteroid causes a dwarf phenotype (Schaller,
2003). Another category of dwarf mutants includes plants that constitutively activate pathogen-related genes. For example, the constitutive expresser of pathogenesis related genes (cpr), defense no death (dnd) and suppressor NPR1 constitutive 1 (snc1) mutants show constitutive expression of a number of defense genes and defective growth (Bowling et al., 1994, 1997; Yu et al., 1998; Zhang et al., 2003).

The defect in elongation of flower stalks in acl mutants is not accompanied by a general reduction in plant growth, although typical dwarf mutants show size reduction of the whole plant body (Tsukaya et al., 1993). According to the metamer concept (Schultz and Haughn, 1991), acl1 and acl2 mutants are unique with respect to their defect in the nodes of type 3 metamers and internode elongation in type 2 metamers, respectively (Tsukaya et al., 1993, 1995). These mutants also display the feature that defective growth can be rescued by rearing the plants at a high temperature (28°C). This feature is usually observed in mutants that show a dwarf phenotype by activation of pathogen-related genes. The bonsai 1 mutant (bon1) shows a constitutive defense response and reduced plant size; this phenotype was exhibited at 22°C but not at 28°C (Yang and Hua, 2004). The cpr30 mutant also shows a temperature-dependent phenotype (Gou et al., 2009). An acl5 mutant was also isolated and this mutant shows a severe reduction in the length of stem internodes and a reduction in the number of flowers due to early proliferative arrest of apical inflorescence meristems (Hanzawa et al., 1997). In addition, acl3 and acl4 mutants were also isolated (Y. Komeda, unpublished data). The gene of the ACL5 locus encodes a spermine synthase, and it was shown that polyamine plays an essential role in the promotion of internode elongation (Hanzawa et al., 2000).

In this study, we detected bidirectional non-coding transcripts from the ACL2 locus. It is highly probable that small RNAs are produced from these non-coding RNAs. Furthermore, transcription of many genes, including genes for the plant immune response, was activated in flower stalks of the acl mutant. We propose a mechanism by which the ACL2 locus regulates elongation of flower stalks.

**MATERIALS AND METHODS**

**Plant materials and growth conditions** Arabidopsis thaliana (L.) Heynh. accession Columbia was used. The acl2 mutant was isolated previously (Tsukaya et al., 1995). The T-DNA insertion lines Salk_057149, Salk_006746 and Salk_088254, which are ed31, pad4 and sid2 mutants, respectively, were obtained from ABRC (Columbus, OH, USA). Plants were grown on rock wool and vermiculite or on Murashige-Skoog (MS) medium containing 1% sucrose and 0.8% agar at 22°C under continuous light.

**Genomic DNA sequences of wild-type and acl2 mutant plants** The 320-kb region containing the ACL2 locus was amplified as approximately 15-kb overlapping fragments by PCR using genomic DNAs of wild-type and acl2 mutant plants as templates. PCR was carried out with LA-Taq (Takara Bio, Otsu, Japan). The sequences of the amplified DNA fragments were directly determined with BigDye Terminator v.3.1 (Applied Biosystems, Foster, CA, USA) by the primer-walking method. Some regions for which sequences could not be determined using PCR products were cloned in plasmid vectors, and sequences of more than three independent clones were determined. Nucleotide sequences were compared using DNASIS sequence analysis software (Takara Bio).

**Complementation test** DNA fragments used to construct plasmids for complementation of the acl2 mutant were amplified using primers with sequences corresponding to 21868240–21868264 and 21873634–21873655 on chromosome 1 according to the AGI map. PCR was carried out with PfuUltra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA, USA). After adding A residues at the 3’ termini using rTaq (Takara Bio) and dATP, the fragments were ligated to the XcmI site in the p3T vector (MoBiTec, Göttingen, Germany). PCR and cloning fidelity was confirmed by DNA sequencing. The resultant plasmids were digested with EcoRI alone (one site is located in the vector and a second in the inserted DNA), EcoRI (located in the vector) and BglII (located in the inserted DNA), or SalI (located in the inserted DNA) and EcoRI (located in the inserted DNA) (see Fig. 1B). Excised fragments were replaced with sequences of the GUS gene and NOS terminator in pBI101 (Clontech, Mountain View, CA, USA). The resultant plasmids were introduced into Agrobacterium tumefaciens strain C58 by the freeze-thaw method (Holsters et al., 1978) and then transformed into acl2 mutant plants by vacuum infiltration (Bechtold and Pelletier, 1998). T1 transformants were selected by resistance to kanamycin, and we confirmed that the introduced DNA fragment was present in transformants by PCR analysis of their genomic DNA.

**RNA extraction** Total RNAs for RT-PCR and microarray analysis were prepared using a Total RNA Isolation Kit (Takara Bio) and treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA). Total RNAs used to extract small RNAs were prepared by TRIzol Reagent (Life Technologies, Rockville, MD, USA), and RNA was recovered by ethyl alcohol precipitation.

**RACE analysis** 3’-RACE was performed using the 3’-Full RACE Core Set (Takara Bio). PCR was performed twice using appropriate primers (Supplementary Table
**Control mechanism of flower stalk elongation**

PCR products were detected by Southern hybridization. The fragment from 21871709 (SalI) to 21872842 (BglII) in chromosome 1 on the AGI map was used as a probe. Probe labeling and detection were performed using the Gene Images Random-Prime Labeling and Detection System (GE Healthcare, Little Chalfont, UK). RACE products of the appropriate sizes were extracted from the agarose gel, and the purified fragments were cloned into the p3T vector. Clones having plasmids that hybridized to the probe used in Southern hybridization were selected by colony hybridization, and sequences of their inserted DNAs were determined.

**Detection of small RNAs** Large-sized RNA was removed from total RNA by LiCl precipitation. The small RNA fraction was recovered from the supernatant of LiCl treatment by ethyl alcohol precipitation. RNAs with sizes of approximately 20 to 40 nucleotides were extracted from the small RNA fraction using the Flash PAGE Fractionator System (Life Technologies). After bacterial alkaline phosphatase treatment, small RNAs were ligated to a 3’-adaptor sequence by T4 RNA ligase, and then small RNAs ligated to the 3’-adaptor were separated and recovered using a denaturing polyacrylamide gel containing 50% urea. The 5’ termini of adaptor-ligated small RNAs were phosphorylated by T4 polynucleotide kinase and ligated to a 5’-adaptor by T4 RNA ligase. Ligated products were again separated and recovered using a denaturing polyacrylamide gel. The resultant fragments comprising the 5’-adaptor, small RNA and the 3’-adaptor were subjected to PCR. Forward primers used a chimeric sequence of the 5’-adaptor and several nucleotides of the 5’-side of small RNAs, and the reverse primer used the sequence of the 3’-adaptor. PCR products were separated by 15% denaturing polyacrylamide gel electrophoresis and electroblotted onto a Hybond N+ filter (GE...
Healthcare) (1 × TB-buffer, 350 mA, 1 h). Membranes were hybridized with oligonucleotide probes labeled using a Gene Images 3'-oligolabeling kit (GE Healthcare). Hybridized membranes were washed three times with 5 × SSC (0.75 M NaCl, 0.075 M trisodium citrate) containing 0.1% SDS for 5 min at room temperature and twice with 3 × SSC containing 0.1% SDS for 15 min at 42°C. Hybridization signals were detected by the CDP-star detection reagent (GE Healthcare). Sequences of primers, adaptors and probes are listed in Supplementary Table S2.

**Microarray analysis** RNAs extracted from stems immediately after bolting in wild-type and acl2 mutant plants were labeled with cyanine3 and cyanine5, respectively. Labeled RNAs were hybridized to the Arabidopsis 3 oligo microarray (Agilent Technologies, Santa Clara, CA, USA). RNA labeling, hybridization, detection and data analysis were carried out by Hokkaido System Science (Sapporo, Japan). The experiment was performed only once.

**RT-PCR analysis** Reverse transcription was performed using a PrimeScript II 1st-strand cDNA Synthesis Kit (Takara Bio). cDNAs were amplified using appropriate primers (Supplementary Table S3), and RT-PCR products were evaluated by agarose gel electrophoresis.

**RESULTS**

**Detection of the mutation point of acl2 and complementation test** The ACL2 locus has been reported to be at 100 map units on chromosome 1 (Tsukaya et al., 1995) and is located within a contig DNA constructed from four P1 clones (Kato et al., 1999) and one BAC clone, named T4M14. The sequences of this region in wild-type and acl2 mutant plants were obtained. One nucleotide substitution from G to A in acl2 mutant plants was detected at 21872231 on the AGI map, and three transgenic lines showed stems with lengths longer than those in the acl2 mutant plants. Furthermore, 16 independent transgenic plants containing the SaII-EcoRI fragment were obtained, and three transgenic lines showed a wild-type-like phenotype. In one line of transgenic plants containing the EcoRI-Bg/II fragment and two lines containing the SaII-EcoRI fragment, the stem lengths were almost the same as those of wild-type plants and the acl2 mutant phenotypes were almost recovered (Fig. 1, D and E). Complementation was not always complete, and the ratio of lines with a recovered acl2 phenotype to independent transgenic lines was much lower than that of a general complementation test. However, if recovery of the acl2 phenotype found in transgenic plants had occurred by an additional mutation to suppress the acl2 phenotype, the observed ratio of transgenic lines showing a recovered phenotype (approximately 17%) was too high. It is thus highly probable that substitution from G to A at 21872231 in chromosome 1 caused the acl2 mutation.

**Detection of RNA products containing the mutation point** Results of the complementation test suggested that information in the DNA sequences containing the acl2 mutation point affects the growth of flower stalks in a trans-acting manner. On the other hand, the TAIR database revealed that no putative genes and no transcripts contain the mutation point. Therefore, we carried out 3'-RACE to detect transcripts containing the mutation point (Fig. 2, A and B). After the second PCR amplification, PCR products of both orientations were observed as smeared bands (Fig. 2A). To confirm that these products were derived from transcripts of sequences around the acl2 mutation point, several clones having PCR products were analyzed. Their sequences revealed that RNAs of both orientations were transcribed from the region around the acl2 mutation point; that the RNAs had a poly(A) sequence at their 3' terminus; and that they had variable 3' ends (Fig. 2B). Next, we tried a 5'-RACE experiment using the CapFishing Full-length cDNA Premix Kit (Seegene, Seoul, Korea), but we failed to detect discrete bands. We could therefore not determine the whole sequences of RNAs transcribed from the ACL2 locus. However, transcripts of both orientations containing the mutated region of acl2 actually existed, and it is highly probable that these transcripts contribute to the acl2 phenotype.

It is possible that these bidirectional transcripts form double-stranded RNAs (dsRNAs) and produce small RNAs. To search for small RNAs corresponding to the region around the acl2 mutation point, we used the Plant MPSS database (Nakano et al., 2006) and the Arabidopsis Small RNA Project (ASRP) website (Backman et al., 2008). Many small RNA molecules existed in the region close to the acl2 mutation point (Fig. 2C). These small RNAs were produced from both strands of DNA and they showed roughly equal populations (Fig. 2D). Figure 2E shows sequences of the majority of small RNAs recorded in the Plant MPSS database. Several small RNAs of both orientations occurred near the acl2 mutation point,
Fig. 2. RNA products transcribed from around the acl2 mutation point. (A) 3'-RACE products of both orientations, revealed by staining with EtBr (lanes 1 and 3) and by Southern hybridization (lanes 2 and 4). Reverse primers (lanes 1 and 2) and forward primers (lanes 3 and 4) in Supplementary Table S1 were used. (B) The thick line shows the genome sequence and the arrowhead indicates the acl2 mutation point. Nucleotide numbers are relative to the acl2 mutation point (+1). Red boxes (using reverse primers) and blue boxes (using forward primers) with poly(A) show 3'-RACE products of both orientations of transcripts. (C) Gene organization and location of small RNAs from the plant ASRP database. Numbers in the top gray box indicate nucleotide positions in chromosome 1 on the AGI map. Red boxes indicate putative protein-coding genes. The histogram was generated by counting small RNA sequences from both strands at each nucleotide position. The arrowhead indicates the acl2 mutation point, and nucleotide numbering corresponds to that in (B). (D) Gene organization and location of small RNAs from the Plant MPSS database. The histograms show the populations of top-strand small RNAs (upper) and bottom-strand small RNAs (lower) in each 100-bp region. The arrow indicates the acl2 mutation point. (E) Nucleotide sequence around the acl2 mutation point and small RNAs in the reverse direction (red) and forward direction (blue). The shadowed G is mutated to A in the acl2 mutant, and nucleotide numbers are relative to this acl2 mutation point at +1.
and we analyzed their existence in several organs in wild-type and acl2 mutant plants (Fig. 3). Since the quantities of these small RNAs were very small (less than 0.7% of miRNA167a in the Plant MPSS database), we used the combination of RT-PCR and Southern hybridization. Four small RNAs, named S-2C/S-2A, S-3, A-1 and A-2, containing the acl2 mutation point were clearly detected in stems and/or rosette leaves, although they were barely detectable in seedlings. All of them except for A-2 were expressed more strongly in stems than in rosette leaves, and their expression in wild-type stems was higher than that in acl2 mutant stems. Since the lengths of probes were approximately 20 nt (Supplementary Table S2), it is possible that the one-nucleotide substitution between the wild type and acl2 mutant affected the efficiency of hybridization. However, the band of S-2 in wild-type stems was stronger than that in acl2 mutant stems even when the sequence of the acl2 mutant was used as a probe (S-2A in Fig. 3). Therefore, the expression of S-2, S-3 and A1 is evidently reduced in stems by the acl2 mutation. Three small RNAs, S-1, S-4 and A-3, were weakly or barely detected in any of the organs.

**Identification of genes whose expression was changed by the acl2 mutation** To try and identify genes that stunt the flower stalks in the acl2 mutant, gene expression in wild-type and acl2 mutant plants was compared using microarray analysis. RNAs were extracted from stems immediately after bolting. Thirty-seven genes exhibited a more than 10-fold difference in expression between wild-type and acl2 mutant plants; 35 of these genes were up-regulated and two were down-regulated in acl2 mutant plants (Table 1). The At1g59580 gene, located approximately 15.5 kb downstream from the acl2 mutation point, showed 7.7-fold higher expression in acl2 mutant plants than in wild-type plants. However, none of the other 19 genes located in the region neighboring the ACL2 locus (from approximately 43 kb upstream to approximately 37 kb downstream) showed more than a 2.0-fold change in expression level. The acl2 mutation therefore appears to have little effect on the expression of genes neighboring the ACL2 locus.

![Fig. 3. Detection of small RNAs in wild-type and acl2 mutant plants. Nucleotide sequences are the genome sequence around the acl2 mutation point (black), small RNAs in the reverse direction (red) and those in the forward direction (blue). Each RNA was amplified by RT-PCR and detected by Southern hybridization. S-2C and S-2A indicate the wild-type sequence and the mutation sequence, respectively, and probes used in Southern hybridization were different (Prob-S-2C and Probe-S2A in Supplementary Table S2 were used for S-2C and S-2A, respectively), although the same RT-PCR primer was used. Se, St and RL indicate that the RNAs used were extracted from seedlings, stems and rosette leaves, respectively. RNAs were extracted from wild-type (W) and acl2 mutant (a) plants. The miR167a sequence was used as a control.](image-url)
Since the microarray analysis was carried out only once, the above changes in gene expression were examined by RT-PCR analysis. Using 15 of the up-regulated genes and the two down-regulated genes in acl2 mutant plants, this analysis confirmed the data obtained from microarray analysis (Fig. 4A). The relative band intensity...

### Table 1. Genes whose mRNA levels differed by more than 10-fold between wild type and acl2 mutant

| Gene ID     | Gene Name | FC (acl2/Col) | Description                                                                 |
|-------------|-----------|---------------|-----------------------------------------------------------------------------|
| At3g50770   | CML-41    | 72.7          | Calmodulin-like 41                                                         |
| At3g57260   | BGL2, PR2 | 53.8          | Beta-1,3-glucanase, Pathogenesis-related protein 2, Plant defense            |
| At5g4200    | PRLIP2    | 51.2          | Alpha/beta-hydrolases super family, Triglyceride lipase activity, induced by SA |
| At3g57240   | BG3       | 41.2          | Beta-1,3-glucanase, Pathogenesis-related                                   |
| At5g10380   | RING1     | 35.3          | Ring finger domain protein with E3 ligase activity, Upregulation by pathogen |
| At3g47480   |           | 32.2          | Calcium-binding EF-hand family protein                                     |
| At2g18690   |           | 30.6          | unknown                                                                    |
| At1g58225   |           | 24.6          | unknown                                                                    |
| At2g18660   | PNP-A     | 23.6          | Plant natriuretic peptide A, Systemically mobile molecule                  |
| At3g45860   | CRK4      | 21.4          | Cysteine-rich receptor-like protein kinase, Induction by pathogen infection  |
| At2g3570    | CHI, AED15| 18.0          | Chitinase activity, APOPLASTIC EDS1-DEPENDENT 15 protein                   |
| At2g41100   | CAL4, TOUCH3 | 16.9     | Calmodulin-like protein, Expression induced by touch and darkness           |
| At2g17040   | ANAC036   | 15.6          | NAC transcription factor family, Dwarf phenotype in overexpressing plants   |
| At5g10760   | AED1      | 15.6          | Asparatic-type endopeptidase, APOPLASTIC EDS1-DEPENDENT 1 protein           |
| At3g28580   |           | 15.5          | AAA-ATPase, P-loop containing triphosphate hydrolases superfamily           |
| At2g20142   |           | 14.4          | TIR domain protein, Transmembrane receptor activity                        |
| At4g02380   | ATLEA5, SAG21 | 13.8    | Late embryogenesis abundant like 5, Senescence-associated gene 21           |
| At3g17890   | CNGC19    | 13.5          | Cyclic nucleotide gated channel 19                                         |
| At5g55460   | ATLTP4.5  | 13.1          | Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily |
| At5g52760   |           | 12.8          | Copper transport protein                                                   |
| At2g31880   | EVR, SOBIR1| 12.6          | Leucine rich repeat transmembrane, Positive regulator of defense signaling  |
| At2g33430   | SPP2      | 12.5          | Sucrose-6F-phosphate phosphohydrolase 2                                     |
| At5g54490   | PBP1      | 12.1          | Pinoid-binding protein, EF-hand calcium-binding protein                      |
| At1g14870   | PCR2      | 12.1          | Plant cadmium resistance 2, Zinc transport and detoxification               |
| At1g90980   | BIP3      | 11.8          | Binding protein 3, ATP binding, Protein folding, Response to heat           |
| At5g64000   | SAL2      | 11.5          | 3'(2'), 5'-bisphosphate nucleotidase                                       |
| At3g60420   |           | 11.4          | Phosphoglycerate mutase family                                             |
| At5g22910   |           | 11.3          | ATPase E1-E2 family, Calcium-transporting ATPase activity                   |
| At3g26830   | PAD3      | 11.0          | Cytochrome P450 involved in the biosynthesis of camalexin                   |
| At5g22380   | NAC090    | 10.9          | NAC domain containing protein 90, DNA binding transcription factor          |
| At5g64120   | PRX71     | 10.7          | Peroxidase 71, Cell wall bound peroxidase, Lignification of cell walls      |
| At1g35170   | LAG1      | 10.6          | Lipid-sensing domain containing protein                                     |
| At4g14365   | XBAT34    | 10.6          | XB3 ortholog 4, Zinc ion binding, Ankyrin repeat                           |
| At3g31210   | RLP38     | 10.4          | Receptor like protein 38                                                    |
| At1g75040   | PR5       | 10.2          | Pathogenesis-related gene 5, Thaumatin-like protein, Response to pathogens  |
| At2g32140   |           | 9.5           | TIR-X protein, Transmembrane receptor, Defense response                     |
| At4g33390   | ADR1-L1   | 4.8           | Activated disease resistance 1 like 1, NB-LRR immune receptor               |
| At5g37940   |           | 0.084         | Zinc-binding dehydrogenase, Oxidoreductase activity                        |
| At4g11210   | AtDIR14   | 0.096         | Dirigent-like protein family, Disease resistance response                   |

Fold change (FC) means level of expression in the acl2 mutant plants compared to that in wild-type plants in the microarray analysis. The FCs of expression levels of At2g32140 and At4g33390 genes were less than 10-fold; these genes are listed because plants that overexpressed them showed a dwarf phenotype.

Since the microarray analysis was carried out only once, the above changes in gene expression were examined by RT-PCR analysis. Using 15 of the up-regulated genes and the two down-regulated genes in acl2 mutant plants, this analysis confirmed the data obtained from microarray analysis (Fig. 4A). The relative band inten-
sities in RT-PCR analysis did not always coincide with the fold changes shown in Table 1; different sequences were used to detect the transcript in the two analyses, and, while this may account for the inconsistencies, quantitative PCR using several primers would be required for a full analysis. Genes up-regulated in acl2 mutant plants were preferentially expressed in rosette leaves in wild-type plants, and genes down-regulated in acl2 mutant plants were preferentially expressed in stems in wild-type plants (Fig. 4B). Although samples of acl2

| Gene   | Col | St  | acl2 |
|--------|-----|-----|------|
| At3g50770 |     |     |      |
| At3g57260 |     |     |      |
| At5g24200 |     |     |      |
| At3g57240 |     |     |      |
| At5g10380 |     |     |      |
| At3g47480 |     |     |      |
| At2g18690 |     |     |      |
| At1g58225 |     |     |      |
| At2g18660 |     |     |      |
| At3g45860 |     |     |      |
| At2g43570 |     |     |      |
| At2g41100 |     |     |      |
| At2g17040 |     |     |      |
| At5g10760 |     |     |      |
| At3g28580 |     |     |      |
| At2g32140 |     |     |      |
| At4g33300 |     |     |      |
| At5g37940 |     |     |      |
| At4g11210 |     |     |      |
| RiboPro-S2 |     |     |      |
stems may have contained some cauline leaf contamination, since the acl2 stems were very short, the results obtained using stems immediately after bolting (Fig. 4A) were similar to those obtained using stems after several flowers had bloomed (Fig. 4B). Therefore, it is highly probable that various genes activated in rosette leaves of wild-type plants failed to be repressed in stems of acl2 mutant plants and that genes activated in stems of wild-type plants failed to be activated in stems of acl2 mutant plants.

Partial suppression of the acl2 mutant phenotype by sid2 mutation Microarray analysis showed that some genes related to the immune system were up-regulated in acl2 mutant plants. It has been reported that an accumulation of salicylic acid often induces a dwarf phenotype (Bowling et al., 1994, 1997; Yu et al., 1998; Yoshioka et al., 2001; Zhang et al., 2003). Therefore, we examined whether several mutations of salicylic acid pathway genes affect acl2 mutant phenotypes. sid2/acl2 double mutant plants showed longer flower stalks than those of acl2 single mutant plants, although they were shorter than those of wild-type plants (Fig. 5). These results suggest that the acl2 mutant phenotype is partially caused by activation of the salicylic acid pathway. We also produced eds1/acl2 and pad4/acl2 double mutant plants, but recovery of the acl2 mutant phenotype was not observed (data not shown).

DISCUSSION

In this study, we analyzed the role of the ACL2 locus in control of flower stalk elongation. Most of the flower stalks in the acl2 mutant are reduced in length (Tsukaya et al., 1995). The ACL2 locus transcribes bidirectional non-coding RNAs and it is highly probable that these non-coding RNAs regulate the elongation of flower stalks.

Complementation efficiency of acl2 mutants We carried out a complementation test for acl2 mutant plants by introducing wild-type DNA fragments of the ACL2 locus. About 17% of the transgenic plants showed a partially or almost completely recovered phenotype. Although the reason for the low efficiency of complementation is not known, we propose the following explanation. In wild-type plants, bidirectional non-coding RNAs transcribed from the ACL2 locus form completely base-paired dsRNA. In contrast, the transgenic plants used in the complementation test transcribe both wild-type RNAs and mutant-type RNAs, and the population of completely base-paired dsRNA is therefore decreased. Small RNAs may be efficiently produced from completely base-paired dsRNA. In this hypothesis, acl2 mutant plants in which DNA fragments of the wild type had been introduced cannot fully produce small RNAs derived from the ACL2 locus. Only transgenic plants that transcribe a large quantity of non-coding RNAs from the introduced DNA fragments can produce sufficient small RNAs and recover the acl2 phenotype. This hypothesis is compatible with the results of a study showing that the acl2 mutant has a semidominant defect in the elongation of flower stalks (Tsukaya et al., 1995). In this study, we analyzed only acl2 mutant plants containing wild-type DNA fragments of the ACL2 locus. Experiments using other transgenic plants, such as wild-type plants in which the acl2-mutated DNA fragment was introduced or plants overexpressing the ACL2 locus under the 35S promoter, will be required to clarify the function of the ACL2 locus.

Difference in small RNAs between wild-type plants and acl2 mutant plants The results of this study suggested that small RNAs derived from the ACL2 locus control the expression of many genes and affect the elongation of flower stalks. The quantity of three small RNAs (S-2, S-3 and A-1) appeared to be larger in stems than in rosette leaves. These small RNAs may function to repress the expression of some genes in flower stalks. This speculation is compatible with the acl2 phenotype, in which flower stalks were very short while the rosette leaves were almost the same size as those of wild-type plants, and also with results showing that genes up-regulated in stems of acl2 mutant plants were preferentially expressed in rosette leaves of wild-type plants.

What difference between the small RNAs in wild-type
The quantity of small RNAs (S-2, S-3 and A-1) seemed to be decreased by acl2 mutation. Why a difference of only one nucleotide would cause a change in their quantities is not known. However, it is possible that reduction of the quantity of these small RNAs caused the failure of repression of some genes in stems of acl2 mutant plants. Another possibility is that small RNAs with a one-nucleotide mutation lose the ability to repress some genes. It has been reported that a one-nucleotide mutation affects the functions of microRNA (Guo et al., 2008; Liu et al., 2014).

### Genes repressed by small RNAs

Many studies on gene expression regulation using microRNAs and various types of small interfering RNAs (siRNAs) have been conducted (reviewed by Bologna and Voinnet, 2014). The various types of siRNAs include cis-natural antisense-siRNAs (cis-nat-siRNAs), which are produced from sense transcripts and cis-natural antisense transcripts (cis-nat) (Zhang et al., 2013). More than a thousand potential cis-nat pairs have been detected in the Arabidopsis genome (Wang et al., 2005) and at least 84 of them could generate siRNAs (Zhang et al., 2012). It was also reported that cis-nat-siRNAs are specific to a particular tissue (Ron et al., 2010) or to environmental stress (Borsani et al., 2005; Katiyar-Agarwal et al., 2007). These reports suggest that cis-nat-siRNAs play an important role in plant development and adaptation to the environment. Although previously reported cis-nat-siRNAs were generated from protein-coding regions, it is possible that they are also produced from intergenic regions such as the ACL2 locus and play various roles in plants.

How the small RNAs identified in this study affect gene expression is not known. Many positions at which sequences were similar to those of small RNAs were detected in the whole genome, but we did not detect sequences similar to those of small RNAs around the genes whose expression was up-regulated in acl2 mutant plants. It is possible that the up-regulated genes in acl2 mutant plants identified in this study are not direct targets of small RNAs and that their transcription is activated by other factors that are direct targets of small RNAs. Identification of the true targets of small RNAs generated from the ACL2 locus is required.

### Relationship between flower stalk elongation and up-regulated genes in acl2 mutants

Transcription of many genes was up-regulated in the flower stalks of acl2 mutants. Which among these genes prevent elongation of flower stalks is unknown at present. However, wild-type plants overexpressing several individual genes that were up-regulated in acl2 mutant plants showed a dwarf phenotype. Overexpression of the ANAC036 gene (At2g17040), whose expression level was 15.6-fold higher in acl2 mutant plants than in wild-type plants, results in a dwarf phenotype (Kato et al., 2010); overexpression of the TIR-X gene (At2g32140), with the expression level being 9.5-fold higher in acl2 mutant plants than in wild-type plants, results in a dwarf phenotype (Kato et al., 2014); and overexpression of the ADR1-L1 gene (At4g33300), with the expression level being 4.8-fold higher in acl2 mutant plants than in wild-type plants, also results in a dwarf phenotype (Kato et al., 2011). These overexpressing plants showed not only short flower stalks but also small rosette leaves. In contrast, acl2 mutant plants showed only stunted flower stalks. The difference in phenotypes may be caused by the difference in organs in which these genes are overexpressed. In plants overexpressing genes under the 35S promoter, the genes were transcriptionally activated in the whole plant, but in acl2 mutant plants, the same genes were transcriptionally activated only in flower stalks. Therefore, the up-regulated genes may affect only flower stalks in acl2 mutants. The TIR-X and ADR-L1 genes are pathogen-related genes, and constitutive activation of pathogen-related genes generally causes dwarf phenotypes in plants (Clarke et al., 1998; Shah et al., 2001; Yoshioka et al., 2001; Shirano et al., 2002; Gil et al., 2005; Gou et al., 2009; Miura et al., 2010). The dwarf phenotypes caused by activation of pathogen-related genes are usually temperature-sensitive; these phenotypes were rescued by a high temperature (28°C). Similar temperature dependency was observed in the acl2 phenotype (Tsukaya et al., 1995). Moreover, acl2 and sid2 double mutant plants, which are defective in salicylic acid synthesis, showed partial recovery of the stunted phenotype of flower stalks displayed by the acl2 mutant plants. Therefore, it is possible that activation of an immune response in flower stalks in acl2 mutant plants prevents the elongation of flower stalks.

### Regulation of flower stalk elongation

In A. thaliana, flower stalks start to elongate after transition to the reproductive phase from the vegetative phase. This developmental process suggests that the elongation of flower stalks is strictly controlled. To analyze this control mechanism, several mutants with stunted flower stalks have been obtained and characterized. The mutation of acl1 affects the development of both inflorescences and leaves (Tsukaya et al., 1993), and it was suggested that the expression of multiple genes can enhance the acl1 phenotype (Kamata and Komeda, 2008). It was also reported that the ACL5 gene, which is required for flower stalk elongation, encodes a spermine synthase (Hanzawa et al., 2000). The results of the present study suggested the following novel mechanism for control of flower stalk elongation (Fig. 6). Bidirectional transcripts are expressed from the ACL2 locus and small RNAs are produced from them. These small RNAs repress the expression of var-
they form dsRNAs, and then small RNAs are produced. The directional non-coding RNAs are transcribed from the mutant phenotypes are partially recovered by the plants. Pathogen-related genes including their transcription is activated by salicylic acid. Therefore, involved in stunting of flower stalks do not accumulate, since

Fig. 6. Diagram of the putative role of the ACL2 locus. Bidirectional non-coding RNAs are transcribed from the ACL2 locus; they form dsRNAs, and then small RNAs are produced. The small RNAs repress the transcription of various genes including At2g32140 and At4g33300, although it is not known which genes are direct targets of small RNAs. Some of the genes whose expression is repressed by small RNAs prevent flower stalk elongation. In the acl2 mutant, a reduction of small RNAs and/or a change in their activity results in the accumulation of transcripts from genes whose expression represses flower stalk elongation. Therefore, flower stalks are stunted in acl2 mutant plants. Pathogen-related genes including At2g32140 and At4g33300 are transcriptionally activated by salicylic acid, and SID2 is required for accumulation of salicylic acid. In acl2/sid2 double mutant plants, transcripts of some genes that are involved in stunting of flower stalks do not accumulate, since their transcription is activated by salicylic acid. Therefore, acl2 mutant phenotypes are partially recovered by the sid2 mutation.

Fig. 6. Diagram of the putative role of the ACL2 locus. Bidirectional non-coding RNAs are transcribed from the ACL2 locus; they form dsRNAs, and then small RNAs are produced. The small RNAs repress the transcription of various genes including At2g32140 and At4g33300, although it is not known which genes are direct targets of small RNAs. Some of the genes whose expression is repressed by small RNAs prevent flower stalk elongation. In the acl2 mutant, a reduction of small RNAs and/or a change in their activity results in the accumulation of transcripts from genes whose expression represses flower stalk elongation. Therefore, flower stalks are stunted in acl2 mutant plants. Pathogen-related genes including At2g32140 and At4g33300 are transcriptionally activated by salicylic acid, and SID2 is required for accumulation of salicylic acid. In acl2/sid2 double mutant plants, transcripts of some genes that are involved in stunting of flower stalks do not accumulate, since their transcription is activated by salicylic acid. Therefore, acl2 mutant phenotypes are partially recovered by the sid2 mutation.

ious genes, especially genes related to the immune response, in flower stalks, and the down-regulation of these genes permits elongation of flower stalks. To confirm this mechanism, direct targets of small RNAs derived from the ACL2 locus must be determined. Further analysis of the mechanism should provide insights into not only the developmental processes of plants and regulatory mechanisms of the plant immune system but also novel roles of small RNAs in plants.

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REFERENCES

Backman, T. W., Sullivan, C. M., Cumbie, J. S., Miller, Z. A., Chapman, E. J., Fahigren, N., Givan, S. A., Carrington, J., and Kasschau, K. D. (2008) Update of ASRP: the Arabidopsis small RNA project database. Nucleic Acids Res. 36, D982–D985.

Bechtold, N., and Pelletier, G. (1998) In planta Agrobacterium-mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration. Methods Mol. Biol. 92, 259–266.

Bologna, N. G., and Voinnet, O. (2014) The diversity, biogenesis, and activities of endogenous silencing small RNAs in Arabidopsis. Annu. Rev. Plant Biol. 65, 473–503.

Borsani, O., Zhu, J., Verslues, P. E., Sunkar, R., and Zhu, J. K. (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. Cell 123, 1279–1291.

Bowling, S. A., Guo, A., Cao, H., Gordon, A. S., Klessig, D. F., and Dong, X. (1994) A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. Plant Cell 6, 1845–1857.

Bowling, S. A., Clarke, J. D., Liu, Y., Klessig, D. F., and Dong, X. (1997) The cpr5 mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell 9, 1573–1584.

Claeys, H., De Bodt, S., and Inzé, D. (2014) Gibberellins and DELLAs: central nodes in growth regulatory networks. Trends Plant Sci. 19, 231–239.

Clarke, J. D., Liu, Y., Klessig, D. F., and Dong, X. (1998) Uncoupling PR gene expression from NPR1 and bacterial resistance: characterization of the dominant Arabidopsis cpr6-1 mutant. Plant Cell 10, 557–569.

Gil, M. J., Coego, A., Mauch-Mani, B., Jordá, L., and Vera, P. (2005) The Arabidopsis csb3 mutant reveals a regulatory link between salicylic acid-mediated disease resistance and the methyl-erythritol 4-phosphate pathway. Plant J. 44, 155–166.

Gou, M., Su, N., Zheng, J., Huai, J., Wu, G., Zhao, J., He, J., Tang, D., Yang, S., and Wang, G. (2009) An F-box gene, CPR30, functions as a negative regulator of the defense response in Arabidopsis. Plant J. 60, 757–770.

Gou, X., Gui, Y., Wang, Y., Zhu, Q. H., Hellman, C., and Fan, L. (2008) Selection and mutation on microRNA target sequences during rice evolution. BMC Genomics 9, 454.

Hanzawa, Y., Takahashi, T., and Komeda, Y. (1997) ACL5: an Arabidopsis gene required for internodal elongation after flowering. Plant J. 12, 863–874.

Hanzawa, Y., Takahashi, T., Michael, A. J., Burtin, D., Long, D., Pineiro, M., Coupland, G., and Komeda, Y. (2000) ACAULIS5, an Arabidopsis gene required for stem elongation, encodes a spermine synthase. EMBO J. 19, 4248–4256.

Hedden, P., and Kamiya, Y. (1997) Gibberellin biosynthesis: enzymes, genes and their regulation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 431–460.

Holsters, M., de Waele, D., Depicker, A., Messens, E., van Montagu, M., and Schell, J. (1978) Transfection and transformation of Agrobacterium tumefaciens. Mol. Gen. Genet. 163, 181–187.

Kamata, N., and Komeda, Y. (2008) An inversion identified in acl1-1 mutant functions as an enhancer of the acl1-1 phenotype. Genes Genet. Syst. 83, 293–300.

Katiyar-Agarwal, S., Gao, S., Vivian-Smith, A., and Jin, H. (2007) A novel class of bacteria-induced small RNAs in Arabidopsis. Genes Dev. 21, 3123–3134.

Kato, A., Suzuki, M., Kawanaka, A., Ooe, H., Higano-Inaba, K., and Komeda, Y. (1999) Isolation and analysis of cDNA within a 300 kb Arabidopsis thaliana genomic region located around the 100 map unit of chromosome 1. Gene
239, 309–316.
Kato, H., Motomura, T., Komeda, Y., Saito, T., and Kato, A. (2010) Overexpression of the NAC transcription factor family gene ANAC036 results in a dwarf phenotype in Arabidopsis thaliana. J. Plant Physiol. 167, 571–577.
Kato, H., Shida, T., Komeda, Y., Saito, T., and Kato, A. (2011) Overexpression of the activated disease resistance 1-like 1 (ADR1-L1) gene results in a dwarf phenotype and activation of defense-related gene expression in Arabidopsis thaliana. J. Plant Biol. 54, 172–179.
Kato, H., Saito, T., Ito, H., Komeda, Y., and Kato, A. (2014) Overexpression of the TIR-X gene results in a dwarf phenotype and activation of defense-related gene expression in Arabidopsis thaliana. J. Plant Physiol. 171, 362–388.
Liu, C., Rennie, W. A., Carmack, C. S., Kanoria, S., Cheng, J., Lu, J., and Ding, Y. (2014) Effects of genetic variations on microRNA: target interactions. Nucleic Acids Res. 42, 9543–9552.
Miura, K., Lee, J., Miura, T., and Hasegawa, P. M. (2010) SIZ1 controls cell growth and plant development in Arabidopsis through salicylic acids. Plant Cell Physiol. 51, 103–113.
Nakano, M., Nobuta, K., Vemaraju, K., Tej, S. S., Skogen, J. W., and Meyers, B. C. (2006) Plant MPSS databases: signature-based transcriptional resources for analyses of mRNA and small RNA. Nucleic Acids Res. 34, D731–D735.
Ron, M., Alandete Saez, M., Eshed Williams, L., Fletcher, J. C., and McCormick, S. (2010) Proper regulation of a sperm-specific cis-nat-siRNA is essential for double fertilization in Arabidopsis. Genes Dev. 24, 1010–1021.
Schaller, H. (2003) The role of sterols in plant growth and development. Prog. Lipid Res. 42, 163–175.
Schultz, E. A., and Haughn, G. W. (1991) LEAFY, a homeotic gene that regulates inflorescence development in Arabidopsis. Plant Cell 3, 771–781.
Shah, J., Kachroo, P., Nandi, A., and Klessig, D. F. (2001) A recessive mutation in the Arabidopsis SS2 gene confers SA- and NPR1-independent expression of PR genes and resistance against bacterial and oomycete pathogens. Plant J. 25, 563–574.
Shirano, Y., Kachroo, P., Shah, J., and Klessig, D. F. (2002) A gain-of-function mutation in an Arabidopsis Toll interleukin1 receptor-nucleotide binding site-leucine-rich repeat type R gene triggers defense responses and results in enhanced disease resistance. Plant Cell 14, 3149–3162.
Tsukaya, H., Naito, S., Rédei, G. P., and Komeda, Y. (1993) A new class of mutations in Arabidopsis thaliana, acaulis1, affecting the development of both inflorescences and leaves. Development 118, 751–764.
Tsukaya, H., Inaba-Higano, K., and Komeda, Y. (1995) Phenotypic characterization and molecular mapping of an acaulis2 mutant of Arabidopsis thaliana with flower stalks of much reduced length. Plant Cell Physiol. 36, 239–246.
Wang, X. J., Gaasterland, T., and Chua, N. H. (2005) Genome-wide prediction and identification of cis-natural antisense transcripts in Arabidopsis thaliana. Genome Biol. 6, R30.
Yang, S., and Hua, J. (2004) A haplotype-specific resistance gene regulated by BONZAI1 mediates temperature-dependent growth control in Arabidopsis. Plant Cell 16, 1060–1071.
Yoshioka, K., Kachroo, P., Tais, F., Sharma, S. B., Shah, J., and Klessig, D. F. (2001) Environmentally sensitive, SA-dependent defense responses in the cpr22 mutant of Arabidopsis. Plant J. 26, 447–459.
Yu, I. C., Parker, J., and Bent, A. F. (1998) Gene-for-gene disease resistance without the hypersensitive response in Arabidopsis dnd1 mutant. Proc. Natl. Acad. Sci. USA 95, 7819–7824.
Zhang, Y., Goritschnig, S., Dong, X., and Li, X. (2003) A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in suppressor of npr1-1, constitutive 1. Plant Cell 15, 2836–2846.
Zhang, X., Xia, J., Li, Y. E., Barrera-Figueroa, B. E., Zhou, X., Gao, S., Lu, L., Niu, D., Chen, Z., Leung, C., et al. (2012) Genome-wide analysis of plant nat-siRNAs reveals insights into their distribution, biogenesis and function. Genome Biol. 13, R20.
Zhang, X., Li, Y., Wu, Z., Polishko, A., Zhang, H., Chinnusamy, V., Lonardi, S., Zhu, J. K., Liu, R., and Jin, H. (2013) Mechanisms of small RNA generation from cis-NATs in response to environmental and developmental cues. Mol. Plant 6, 704–715.