An inversion identified in *acl1-1* mutant functions as an enhancer of the *acl1-1* phenotype

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The *Arabidopsis acaulis1-1* (*acl1-1*) mutant exhibits severe growth defects when grown at 22°C. The leaves are tiny and curled and the inflorescence stems are short. We identified an inversion mutation in the original *acl1-1* plants. The *acl1-1* plants were crossed with Columbia wild-type, and the *acl1-1* phenotype and the inversion were segregated in the F2 generation. Compared to the original *acl1-1* plants with the inversion, the genuine *acl1-1* plants without the inversion grew larger and their inflorescence stems grew longer at 22°C. When the plants were grown at 24°C, the differences in growth became more apparent. We investigated the expression of genes located in the inversion. Two genes that were located at each end of the inversion were disrupted, and full-length transcripts were not expressed. Expressions of some genes within and adjacent to the inversion were also altered. Our results indicate that the expression of multiple genes may be involved in the enhancement of the *acl1-1* phenotype.

Key words: *acaulis1-1* (*acl1-1*), *Arabidopsis thaliana*, inversion, enhancer

INTRODUCTION

The *Arabidopsis acaulis1* (*acl1*) mutant has defects in inflorescence elongation as represented in its name: no (a-) stem (caulis) (Tsukaya et al., 1993). Two *acl1* mutant alleles were isolated in previous studies (Tsukaya et al., 1993). The *acl1-1* mutation was induced by X-ray irradiation and the *acl1-3* mutation by ethyl methane-sulfonate (EMS) treatment. The *acl1-1* plants exhibit severe phenotypes and develop tiny rosettes with curly leaves. The inflorescence stems are so short that only the upper parts of the stems can be seen over the rosette leaves. At the top of the inflorescence, a few fertile flowers are differentiated. The reduction in the size of rosettes and the length of the inflorescence stems are the common phenotype of *acl1* mutants; however, the growth defects in the *acl1-3* plants are more moderate. These growth defects observed in *acl1* mutants are similar to the dwarf phenotypes of phytohormone mutants. Actually, treatments with common phytohormones such as auxin, brassinosteroid, and gibberellin failed to rescue *acl1* phenotypes. The restoration of the *acl1* phenotype occurs when *acl1* plants are grown at higher temperatures, where *acl1* plants became indistinguishable from

wild-type plants. Ambient temperature is known to influence aspects of the appearances of plants, such as leaf size and stem length (Thingnaes et al., 2003, Atkin et al., 2006). However, the dramatic alteration in morphology of the *acl1* phenotype at high temperature cannot be explained by general developmental variations controlled by ambient temperature. This unique phenomenon observed in *acl1* mutants suggests that the *ACL1* gene is novel gene controlling plant morphology at different ambient temperatures.

The cloning of the *ACL1* gene will give us the answer why the *acl1* plant morphology is dependent on temperature. During molecular mapping of *acl1*, we identified an inversion mutation in the original *acl1-1* mutant. The *acl1-1* phenotype was segregated from the inversion, indicating that the inversion was not the *acl1* mutation itself. We obtained the genuine *acl1-1* mutant without the inversion and, carried out thorough analyses of *acl1-1* plants with and without the inversion. The inversion enhances the *acl1-1* phenotype, inhibiting the elongation of inflorescence stems and reducing the size of rosettes. The inversion disrupts two genes located at each end of the inverted region. We analyzed the expression of these two genes and the genes within and adjacent to the inversion to understand the effects of the inversion. Our experimental data suggest that expression of multiple genes may be involved in the enhancement of the *acl1-1* mutation.

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MATERIALS AND METHODS

Plant materials and growth conditions *Arabidopsis thaliana* ecotype Columbia (Col) was used as the wild-type. The original *acl1-1* mutants were isolated as previously described (Tsukaya et al., 1993). Seeds were sown on water-moistened rock wool placed on vermiculite. For synchronous germination, seeds were placed in darkness at 4°C for 3 days before they were transferred to growth chambers. The growth chambers were set at 22, 24, 26, or 28°C under long-day conditions (16 h light/8 h darkness). Plants were watered with medium containing 1.5 mM NaH₂PO₄ 2H₂O, 0.26 mM Na₂HPO₄, 3.0 mM KCl, 0.0118 mM Fe(III)EDTA, 1.5 mM MgSO₄ 7H₂O, 5.0 mM Ca(NO₃)₂, 0.5 mM NH₄Cl, 3.0 μM H₃BO₄, 0.1 μM ZnSO₄ 7H₂O, 1 μM CuSO₄ 5H₂O, 10 μM MnSO₄ 4H₂O, 0.175 μM MoO₃, and 0.13 μM CoCl₂ 6H₂O.

We obtained the transgenic line SGT754-5-3, which has a T-DNA insertion in the 3rd intron of *At4g21960* (Landsberg *erecta* (L-*er*) background), and two other transgenic lines SALK_018861 and SALK_044071, which have T-DNA inserted in the promoter region and 3'UTR of *At4g22250*, respectively (both are Col background, Alonso et al., 2004). The full length of *At4g21960* transcript was undetectable in SGT754-5-3. Transcripts of *At4g22250* in SALK_018861 and SALK_044071 were in detectable level in RT-PCR.

Examination of the inversion Genomic DNA was extracted from rosette leaves as described by Edwards et al. (1991). Primers used for the examination of the existence of genomic region from *At4g22290* to *At4g21690* were designed using sequences more than 50 bp distant from the gene regions annotated by TAIR (<http://www.arabidopsis.org>). Expected PCR fragments were less than 7 Kbp in length, and were amplified with Ex taq (Takara Bio). PCR fragments were then treated with several restriction enzymes and cleaved into shorter fragments to identify the changes in fragment length more precisely by agarose gel electrophoresis. In the case of genes longer than 7 Kbp, genes were separated in two parts to ensure the effective PCR amplification. For the amplification of *At4g21960*, the following primer pair was used: NK215, 5'-GAGATCAGTAAAATAGATCG-3' and NK216, 5'-TTTAAGGAGCGTGCATTGC-3' were used as primers. Primers used to amplify *At4g22250* were NK232: 5'-TATAATGTCTCATCATCTGC-3' and NK240, 5'-TCGAGTATCTCAATGATCGG-3'; and to amplify *At4g21920*, NK248, 5'-AAACATCAAACCTCACGGAG-3' and NK249, 5'-AATACGTAGTTTTGACCTGG-3'. PCR fragments approximately 2.2 Kbp, 0.8 Kbp and 3.3 Kbp in length were obtained by PCR for *At4g21960*, *At4g22250*, and *At4g21920*, respectively. The primers NK255, 5'-AGATCACATTGAATCTGCAG-3' and NK256, 5'-TAAGTCAGTGTGGAACCTAAG-3' were designed from sequences in the

non-gene-coding region between *At4g21960* and *At4g21970* to obtain 1.7 Kbp PCR products. In this study, this primer pair was used only for the positive control of PCR (Fig. 1). To detect the conjugated fragments of *At4g21960* and *At4g22250*, NK215 and NK240 were used to obtain an approximately 1.3 Kbp PCR fragment. NK216 and NK232 were used to obtain an approximately 1.6 Kbp PCR fragment only from the inversed DNA. Conjugated fragments were sequenced with the BigDye® Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems).

A CAPS marker, MASC04642, was designed from a sequence at the polymorphism site between Col and L-*er* ecotypes. PCR was performed with the primer pair MASC04642-1, 5'-ACAGTAATCGCCTACTTGG-3' and MASC04642-2, 5'-ATTGAAATGCTGGCATTGGG-3'. PCR products were treated with *Eco*T14I. Two fragments were obtained from Col genomic DNA (399 bp and 176 bp) and a 575bp fragment was obtained from L-*er* genomic DNA.

Expression analysis Plants were grown for 10 days on the medium described above, supplemented with 0.8% Bacto agar (BD) and 2% sucrose at 22°C under long-day conditions. RNA was extracted using a RNeasy® Plant Mini Kit (Qiagen). Extracted RNA was treated with Cloned DNaseI (Takara Bio). The reverse-transcriptase reaction was carried out with the Random 9 mers to synthesize cDNA from every type of transcript. Both the reverse transcriptase (RT) reaction and the polymerase chain reaction (PCR) were carried out using an RNA PCR kit (AMV) Ver. 3.0 (Takara Bio). Specific primers to amplify transcripts of *At4g21960* were NK244, 5'-ACTGCGCGTGGAGTCATG-3' and NK856, 5'-GTAGCATGTGAGGGACGTGG-3'; for *At4g22250*, NK398, 5'-AGACGGAGATTCCAGGTTG-3' and NK239, 5'-TTAGAGTTTCCGTTACCGAG-3', and for *EF1α* (*At5g60390*, as a control), NK24, 5'-ACTTGCAGCTATGGGTAAAG-3' and NK25, 5'-CGAAAGTCTCATCATTTGGC-3'.

Observation of cellular structure and evaluation of cell length Stems of plants grown for 40 days after germination were fixed overnight in FAA [70% ethanol:formaldehyde:acetic acid = 18:1:1 (v/v)] then dehydrated in ethanol series. Dehydrated samples were submerged in 50% (v/v) Technovit 7100 resin in an ethanol for 1 h then embedded in 100% Technovit resin as described in the manual (Kluzer). Sections (5-μm thick) were stained with 0.05% toluidine blue O dissolved in 1% (w/v) Na₂BO₄O₇ 10H₂O. Cell size was measured on digital camera-photographed images using image analysis software (Adobe Photoshop). The average cell length was calculated within individuals and then average and standard deviation of each strain was determined (Fig. 3). Two individuals were analyzed for the type 2

metamer (Schultz and Haughn, 1991) of the Col +/+ plants at 22°C, the type 2 metamer of the Col +/+ plants at 24°C, the type 2 metamer of the Col +/+ plants at 28°C, the type 3 metamer of the Col *inv/inv* plants at 28°C, and the *acl1-1 inv/inv* plants at 22°C. Five *acl1-1* +/+ plants grown at 22°C were analyzed and four *acl1-1* +/+ plants grown 24°C. Three individuals from each strain and each temperature were used for all other analysis. Ten cells from each individual were assayed to evaluate t-value (Table 3).

RESULTS

Inversion mutation in the original *acl1-1* Previous research showed that the *acl1-1* mutation was mapped on chromosome 4 near *AGAMOUS* (*AG*) (Tsukaya et al., 1993). Our recent result from genetic mapping positioned the *acl1-1* mutation between the CAPS marker G4539 and the polymorphism MASC04642 on chromosome 4. The *acl1-1* mutant was induced by X-ray irradiation and exhibits a more severe phenotype than the EMS-mutagenized *acl1-3* mutant. Thus, we suspected that the chromosomes of the *acl1-1* mutant are seriously disrupted. In order to explore chromosome disruptions, approximately 260 Kbp of the genomic region from *At4g22290*, the nearest gene to MASC04642, to *At4g21690* was examined by amplifying annotated gene regions by PCR. As the result, two PCR products including *At4g21960*, which encodes a peroxidase (Apel and Hirt, 2004, Welinder et al., 2002), and *At4g22250*, which encodes a zinc finger protein (Kosarev et al., 2002), were absent in the original *acl1-1* mutant (Fig. 1, AB). The other PCR products detected had lengths corresponding to those of the wild-type (data not shown). Every PCR product was present in the *acl1-3* mutant, which indicated that the absence of the two PCR products was specific to the *acl1-1* mutant. The two genes are located at a distance of approximately 120 Kbp on the same chromosome, and the genomic regions between them were intact. One possibility was that the genomic region between *At4g21960* and *At4g22250* was inverted. To investigate this possibility, PCR experiments were performed using combinations of primers at *At4g21960* and *At4g22250*. If these two genes are fused to each other, conjugated fragments would be detected. Both combinations of primers examined were successful in amplifying novel DNA fragments from the *acl1-1* plants (Fig. 1C, see Fig. 1D for locations of primers). The conjugated fragments were cloned and their sequences confirmed that *At4g21960* and *At4g22250* were cleaved and fused each other (Fig. 1D). These results suggested that the 120 Kbp region between *At4g21960* and *At4g22250* was inverted in the original *acl1-1* mutant.

We crossed the original *acl1-1* plants to Col wild-type to evaluate the segregation of the *acl1-1* phenotype and

the inversion. The F2 population was segregated into 496 (73.4%) wild-type plants and 180 (26.6%) *acl1-1* plants at a 3:1 ratio ($\chi^2 = 0.716$). We extracted DNA from 371 wild-type plants and 146 *acl1-1* plants, and examined whether these plants carried the inversion (Table 1). Regarding the inversion, the segregation at +/+ : *inv*/+ : *inv/inv* at the ratio of 1:2:1 was less reliable ($\chi^2 0.025 (2) = 7.38 < \chi^2 = 9.07 < \chi^2 0.010 (2) = 9.21$). The *acl1-1* phenotype did not necessarily cosegregate with the inversion. Thus, the inversion is independent of the cause of the *acl1-1* phenotype. The recombination rate between the *acl1-1* phenotype and the inversion was estimated to be 15.4% according to the segregation of the F2 plants. To simplify descriptions, we refer to the newly isolated *acl1-1* plants without the inversion as *acl1-1* +/+, the original *acl1-1* plants with the inversion as *acl1-1 inv/inv*, wild-type plants as Col +/+, and the wild-type phenotype (*ACL1/ACL1*) plants with the inversion as Col *inv/inv*.

Full-length transcripts of *At4g21960* and *At4g22250* were not detected in the *inv/inv* plants (Fig. 1E). We cloned *At4g21960* and *At4g22250* from the *acl1-3* plants and found that there was no mutation. Moreover, the expression levels of these two genes in the *acl1-3* mutants were identical to those in the wild-type (data not shown). Thus, the *acl1-3* phenotype does not require a mutation in *At4g21960* nor *At4g22250*. T-DNA insertion lines in *At4g21960* and *At4g22250* had no obvious growth defects (data not shown). Together our results indicated that the loss of either of *At4g21960* or *At4g22250* does not alter morphology of the plants.

Expressions of genes related to the inversion We investigated the expression of two genes that are located at the fusion-points of in the inversion, *At4g21960* and *At4g22250*. And the expressions of those genes located within and adjacent to the inversion were examined (Supplementary Fig. 1, also see Supplementary Table 1 for gene functions and sequences of primers used for analyses). Expression of some genes differed among the four plant strains: Col +/+, Col *inv/inv*, *acl1-1* +/+, and *acl1-1 inv/inv*. From RT-PCR experiments, we observed a decrease in expression of *At4g22270* (*IMMUTANS*, Wu et al., 1999), an increase in *At4g22214* (defensin like protein, Silverstein et al., 2005), and a slight increase in *At4g22235* (defensin like protein) in two *inv/inv* plants. In the *acl1-1 inv/inv* plants, we found increased expression of *At4g22050* (aspartyl protease family protein) and *At4g22070* (WRKY DNA-BINDING PROTEIN 31, Eulgem et al., 2000) and decreased expression of *At4g21990* (APS REDUCTASE 3, Houston et al., 2005), *At4g22010* (SKU5 SIMILAR 4, Sedbrook et al., 2002) and *At4g22212* (defensin like protein). Among the genes investigated, *At4g22080* (pectate lyase family protein), *At4g22090* (pectate lyase family protein), *At4g22210* (Cys-rich protein),

Fig. 1. Identification of inversion in the original *ac1-1* mutants. A, Locations of genes assayed in this study. B, PCR performed with DNA from wild-type, the original *ac1-1* mutant, and the *ac1-3* mutant to detect full-length genomic regions of *At4g21960* and *At4g22250*. C, Novel conjugated fragments detected in the original *ac1-1* mutant. D, Structure of wild-type *At4g21960* and *At4g22250* and conjugated genes caused in the inversion. Filled and open squares: exons. Bold lines: introns. Arrows with pNK-number: positions of primers used. Within nucleotide sequences, gray shading, *At4g21960*; white boxes, *At4g22250*; capital letters, exons; small letters, introns. Underlined 6 bp were duplicated. Black shading indicates 2 bp substitution from TT to AC. A 34-bp fragment was deleted from *At4g22250*. E, RT-PCR performed with RNA from 10-day-old plants.

Table 1. Segregation of *acl1-1* phenotype and the inversion

Phenotype	Inversion		
	Wild-type (+/+)	Hemizygote (<i>inv</i> /+)	Homozygote (<i>inv/inv</i>)
Wild-type	123	203	45
<i>acl1-1</i>	5	22	119

At4g22217 (defensin like protein) and *At4g22230* (defensin like protein) decreased in both the *acl1-1* plants, regardless of the inversion. The expression of *At4g22030* (F-box family protein) and *At4g22100* (glycosyl hydrolase family 1 protein) was decreased in the Col *inv/inv* plants and the *acl1-1* +/+ plants, and even further decreased in the *acl1-1 inv/inv* plants compared to the Col +/+ plants. The expression patterns of these genes in the *acl1-3* plants were similar to those of the Col +/+ plants. We speculated that the alteration in the expression pattern is due to mutations in *At4g22050*, *At4g22080*, *At4g22090*, and *At4g22270*. Thus, these genes were cloned from *acl1-1* plants and *acl1-3* plants and sequenced. As the results, these genes were identical to the wild-type (data not shown). Furthermore, we found that expression of the partial transcripts from the fused fragments of both *At4g21960* and *At4g22250* was maintained in the *inv/inv* plants (Supplementary Fig. 1B). In the case of *At4g22250*, the partial transcripts were rather increased in the plants with the inversion. Novel transcripts were also detected by RT-PCR using combinations of primers at *At4g21960* and *At4g22250* specific to the plants with the inversion. Thus, the inversion affected expression of a wide range of genes.

The *acl1-1* phenotype was enhanced by the inversion We compared the phenotype of *acl1-1* plants without the inversion to *acl1-1* plants with the inversion. At 22°C, there were no apparent differences between Col

inv/inv plants and Col +/+ plants. The plant heights were identical to each other (Fig. 2A, Table 2). Both the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants exhibited the *acl1-1* phenotype with small curly leaves and short inflorescence stems. However, the comparison of the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants made us realize the difference between these two genotypes. The rosettes of the *acl1-1 inv/inv* plants appeared slightly smaller than those of the *acl1-1* +/+ plants (Fig. 2A). The height of the *acl1-1* +/+ plants was significantly different to that of the *acl1-1 inv/inv* plants, indicating that the growth defects of the *acl1-1* mutants are enhanced by the inversion (Table 2).

We observed sections of inflorescence stems, and found that the *acl1-1* mutation caused serious defects in the length of cells. The reduction of the cell length was significant in *acl1-1* background in all types of tissues examined; cells in the epidermis, the outermost layer of cortex, and the pith, (Table 3). The reduction in the size of the epidermal cells was most severe, and the cells in pith also were significantly affected by the *acl1-1* mutation. The

Table 2. Measurement of plant height at 22°C

Genotype	Average $\pm$ standard deviation (cm)		t-value
<i>acl1-1</i> +/+*	6.0	$\pm$ 1.8	(n = 39)
<i>acl1-1 inv/inv</i> *	4.3	$\pm$ 1.2	(n = 31) t = 4.52 [†]
Col +/+**	179.7	$\pm$ 17.4	(n = 16)
Col <i>inv/inv</i> **	180.0	$\pm$ 29.1	(n = 7) t = 0.031 ^{††}

Plants were grown for 40 days.

* Total plant height including the rosette and the inflorescence stem.

** Length of the inflorescence stem was measured as plant height.

[†] Difference between *acl1-1* +/+ and *acl1-1 inv/inv* is significant (significance level: α = 0.05).

^{††} Col +/+ and Col *inv/inv* are identical (significance level: α = 0.05).

A, 22°C

B, 24°C

Fig. 2. Enhancement of *acl1-1* phenotype by the inversion. A, Overall development of *acl1-1* +/+ plants and *acl1-1 inv/inv* plants was observed 40 days after germination. Rosettes of Col +/+ plants and Col *inv/inv* plants were observed 35 days after germination. Plants were grown at 22°C. Bar = 3 cm. B, Plants were grown at 24°C for 40 days after germination. Bar = 3 cm.

Table 3. Comparison between cell length of +/+ and *inv/inv* plants

	Epidermis (μm)	Cortex (μm)	Pith (μm)	n*
22°C <i>acl1-1</i> +/+	43.0 ± 14.6	28.5 ± 6.38	78.0 ± 25.0	50
22°C <i>acl1-1 inv/inv</i>	21.2 ± 9.25	26.1 ± 6.84	43.6 ± 11.1	20
t-value	6.19 [†]	1.36	5.91 [†]	
22°C Col +/+ 2met**	266.7 ± 100.4	40.2 ± 8.61	175.3 ± 48.9	20
22°C Col <i>inv/inv</i> 2met**	243.2 ± 54.0	38.5 ± 11.9	197.2 ± 39.1	30
t-value	1.07	0.552	-1.75	
22°C Col +/+ 3met**	232.1 ± 54.4	31.2 ± 9.03	178.1 ± 54.6	30
22°C Col <i>inv/inv</i> 3met**	245.9 ± 56.5	31.7 ± 7.96	159.8 ± 43.7	20
t-value	-0.803	-0.209	1.25	
24°C <i>acl1-1</i> +/+ 2met**	112.6 ± 62.3	28.6 ± 8.71	92.7 ± 35.5	40
24°C <i>acl1-1 inv/inv</i> 2met**	25.6 ± 7.24	36.4 ± 10.9	72.1 ± 39.3	20
t-value	6.20 [†]	-2.99 [†]	2.04 [†]	
24°C <i>acl1-1</i> +/+ 3met**	205.0 ± 83.1	31.5 ± 9.56	128.1 ± 31.2	30
24°C <i>acl1-1 inv/inv</i> 3met**	23.2 ± 14.4	13.7 ± 6.30	37.4 ± 18.6	30
t-value	11.81 [†]	8.53 [†]	13.67 [†]	

[†] Difference is significant (significance level: $\alpha = 0.05$).
*Total number of cells assayed for calculate t-value.
**Metameric type of apical meristems: 2met, the type 2 metamer, main inflorescence stem differentiates cauline leaves with elongating internodes; 3met, the type 3 metamer, main florescence stem bears flowers without bracts formed upon the type 2 metamer (Schultz and Haughn, 1991).

acl1-1 inv/inv plants exhibited more severe reduction in the cell length than the *acl1-1 +/+* plants. Unlike cells in epidermis and pith, the length of cortex cells was less affected. In addition to the severe reduction in length the differentiation of cells appeared to be inhibited in the inflorescence stems of the *acl1-1 +/+* plants and the *acl1-1 inv/inv* plants.

The inversion affects *acl1-1* growth at 24°C We observed morphological characteristics under several different temperature conditions. Both the *acl1-1 +/+* plants and the *acl1-1 inv/inv* plants were able to restore their plant morphology when grown at 28°C as previously reported (Tsukaya et al., 1993). Plants tend to become more slender at higher temperatures, as the result of the general effects of higher temperature. These general effects of higher temperature were observed in the Col *inv/inv* plants as well as in the Col +/+ plants throughout our experiments. Correspondingly, the cells became longer at higher temperature (Fig. 3, B and C). Plants were

Fig. 3. Length of cells of inflorescence stems. A, Lengths of cells in epidermis, cortex, and pith were measured and compared to the type 2 metamer of Col +/+ plants. Percentages indicate the ratio to the length of type 2 metamer of the Col +/+ plants. 2met = type 2 metamer, 3met = type 3 metamer. B, C, Length of cells in epidermis of type 2 (B) and type 3 (C) metamer at 22, 24, 26, and 28°C. At 22°C, the type 2 and type 3 metamers of the *acl1-1 +/+* plants and the *acl1-1 inv/inv* plants were assayed together. Lines: averages of each plant strain.

Table 4. Measurement of plant height at 26°C

Genotype	Average $\pm$ standard deviation (cm)		t-value
<i>acl1-1</i> +/+*	227.6	± 27.5	(n = 12) t = -0.350 [†]
<i>acl1-1 inv/inv</i> *	227.3	± 52.1	(n = 8) t = -0.242 [†]
Col +/+*	221.4	± 54.5	(n = 12)
Col <i>inv/inv</i> *	231.0	± 58.7	(n = 16) t = -0.519 [†]

Plants were grown for 40 days.

*Length of the inflorescence stem was measured as plant height.

[†] Height of the plants is identical to Col +/+.

grown at 24 and 26°C to observe morphology at the intermediate temperatures. The restoration of the *acl1-1* phenotype to wild-type was not complete at 24°C (Fig. 2B). However, the difference between the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants became more obvious at 24°C. The inflorescence stems of *acl1-1* +/+ plants elongated to approximately 5 cm in length (average $\pm$ standard deviation, 5.2 ± 3.4 cm, n = 12). On the other hand, the inflorescence stems of *acl1-1 inv/inv* were as short as the plants grown at 22°C. The length of cells, including those in the cortex, became significantly different between the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants (Table 3). Neither the *acl1-1* +/+ plants nor the *acl1-1 inv/inv* plants fully restored the *acl1-1* phenotype at 24°C, however the inflorescence stems of both the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants elongated to a similar length to those of Col plants at 26°C. Complete restoration to wild-type occurred at 26°C, as measured by the length of the epidermal cells in the inflorescence stems (Table 4, Fig. 3, B and C). There was no difference between the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants at temperatures exceeding 26°C (t = 0.014 for plant height at 26°C).

DISCUSSION

In this study, we isolated the “genuine” *acl1-1* plants without the inversion. By comparing these plants to the original *acl1-1* plants with the inversion, we showed that the inversion functions as an enhancer of the *acl1-1* phenotype.

In the previous study, it was reported that the length of the inflorescence stems of *acl1-1* plants varied among plants, even though they were grown side by side under the same conditions at 22°C (Tsukaya et al., 1993). We suggest that this unknown factor that influenced the growth of the original *acl1-1* mutants is the inversion located on chromosome 4. The effects of the inversion on the growth of *acl1-1* plants were evident at 22°C. The height of plants, the size of rosettes, and the length of cells were remarkably reduced in the *acl1-1 inv/inv* plants compared with the *acl1-1* +/+ plants (Tables 2 and 3, Figs. 2A and 3A), thus, the inversion enhances the *acl1-1* phenotype. The inversion functioned more effectively

as an enhancer of the *acl1-1* phenotype when plants were grown at 24°C. The *acl1-1* phenotype is gradually restored to wild-type as temperature increases. The *acl1-1* +/+ plants partially restored the mutant phenotype to wild-type but the *acl1-1 inv/inv* plants did not (Fig. 2B). When the mutant phenotype was fully restored to wild-type in the *acl1-1* plants at 26°C or 28°C, the difference between the *acl1-1* +/+ plants and the *acl1-1 inv/inv* plants disappeared. The inversion itself was not sufficient to alter plant morphology in the wild-type background. It appears that the inversion enhances the defects in the plant growth only when growth defects are already present in *acl1-1* plants.

Many aspects of plant development are affected in *acl1-1* plants. We analyzed expressions of genes within and adjacent to the inversion. In addition to loss of full-length transcripts of *At4g21960* and *At4g22250*, the expression patterns of some other genes were altered (Supplementary Fig. 1). There were several genes that showed remarkable alteration in expression pattern depending on the inversion (e.g. *At4g22270*, *At4g22214*, and *At4g22235*). As the inversion affected the expression of many genes, a complex effect caused by multiple genes may be responsible for the enhancement of the *acl1-1* phenotype. At present, we only analyzed the expression of genes within and adjacent to the inversion using RNA extracted from young seedlings. However, organ-specific and/or age-specific expression of these genes may also be affected by the inversion. To further clarify why the *acl1-1* phenotype is enhanced by the inversion, we will further investigate genes involved in the inversion and their relationship to the *acl1-1* phenotype.

The original *acl1-1* mutant was obtained by X-ray irradiation. In most higher plants, including *Arabidopsis*, DNA double-strand breaks caused by ionizing irradiation (fast neutron, X-ray, and γ -ray) are predominantly repaired by non-homologous end-joining rather than by simple ligation or accurate homologous recombination. In non-homologous end-joining, any end can fuse with any end. Thus, the repair of double-strand breaks in plants is suggested to be error-prone (Gorbunova and Levy, 1999). Sometimes, the repair of double-strands breaks have a very complex DNA rearrangements, combining deletions, insertions, inversions and duplications of the original sequence (Shirley et al., 1992). The inversion isolated from the original *acl1-1* was also complex at the border regions, including deletions, insertions, duplications, and substitutions (Fig. 1D). The *acl1-1* mutation itself would be caused by the error in the repair of double-strand breaks caused by X-ray irradiation. The “genuine” *acl1-1* plants still exhibit more severe growth defects than the *acl1-3* plants induced by EMS (data not shown), which induces base substitutions from G:C to A:T (Colbert et al., 2001). Considering the differences in mutagens

and phenotypes between *acl1-1* and *acl1-3* plants, the *acl1-1* mutation is expected to be a serious DNA rearrangement rather than a simple base substitution.

We are continuing our molecular mapping of *acl1-1* and, in the near future, the details of the *acl1-1* mutation will be revealed.

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REFERENCES

- Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R. et al., (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**, 653–657.
- Apel, K., and Hirt, H. (2004) REACTIVE OXYGEN SPECIES: Metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* **55**, 373–399.
- Atkin, O. K., Loveys, B. R., Atkinson, L. J., and Pons, T. L. (2006) Phenotypic plasticity and growth temperature: understanding interspecific variability. *J. Exp. Bot.* **57**, 267–281.
- Colbert, T., Till, B. J., Tompa, R., Reynolds, S., Steine, M. N., Yeung, A. T., McCallum, C. M., Comai, L., and Heinkoff, S. (2001) High-throughput screening for induced point mutations. *Plant Physiol.* **126**, 480–484.
- Edwards, K., Johnstone, C., and Thompson, C. (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* **19**, 1349–1350.
- Eulgem, T., Rushton, P. J., Robatzek, S., and Somssich, I. E. (2000) The *WRKY* superfamily of plant transcription factors. *Trends Plant Sci.* **5**, 199–206.
- Gorbunova, V., and Levy, A. A. (1999) How plants make ends meet: DNA double-strand break repair. *Trends Plant Sci.* **4**, 263–269.
- Houston, N. L., Fan, C., Xiang, J. Q., Schulze, J. M., Jung, R., and Boston, R. S. (2005) Phylogenetic analyses identify 10 classes of the protein disulfide isomerase family in plants, including single-domain protein disulfide isomerase-related proteins. *Plant Physiol.* **137**, 762–778.
- Kosarev, P., Mayer, K. X., and Hardtke, C. S. (2002) Evaluation and classification of RING-finger domains encoded by the *Arabidopsis* genome. *Genome Biol.* **3**, research0016.1–0016.12.
- Sedbrook, J. C., Carroll, K. L., Hung, K. F., Masson, P. H., and Somerville, C. R. (2002) The *Arabidopsis* *SKU5* gene encodes an extracellular glycosyl phosphatidylinositol-anchored glycoprotein involved in directional root growth. *Plant Cell* **14**, 1635–1648.
- Schultz, E. A., and Haughn, G. W. (1991) *LEAFY*, a homeotic gene that regulate inflorescence development in *Arabidopsis*. *Plant Cell* **3**, 771–781.
- Shirley, B. W., Hanley, S., and Goodman, H. M. (1992) Effects of ionizing radiation on a plant genome: analysis of two *Arabidopsis transparent testa* mutations. *Plant Cell* **4**, 333–347.
- Silverstein, K. A., Graham, M. A., Paae, T. D., and VandenBosch, K. A. (2005) Genome organization of more than 300 *Defensin-Like* genes in *Arabidopsis*. *Plant Physiol.* **138**, 600–610.
- Thingnaes, E., Torre, S., Ernstsens, A., and Moe, R. (2003) Day and night temperature responses in *Arabidopsis*: effects on gibberellin and auxin content, cell size, morphology and flowering time. *Ann. Bot.* **92**, 601–612.
- Tsukaya, H., Naito, S., Redei, 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.
- Welinder, K. G., Justesen, A. F., Kjærsgård, I. V. H., Jensen, R. B., Rasmussen, S. K., Hans, M., Jespersen, H. M., and Duroux, L. (2002) Structural diversity and transcription of class III peroxidases from *Arabidopsis thaliana*. *Eur. J. Biochem.* **269**, 6063–6081.
- Wu, D., Wright, D. A., Wetzela, C., Voytas, D. F., and Rodermel, S. (1999) The *IMMUTANS* variegation locus of *Arabidopsis* defines a mitochondrial alternative oxidase homology that functions during early chloroplast biogenesis. *Plant Cell* **11**, 43–56.