DNA polymorphism at the *ACAULIS5* locus of the wild plant *Arabidopsis thaliana*

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Nucleotide variation in the *ACL5* gene region, which encodes spermine synthase, was analyzed for 21 *Arabidopsis thaliana* ecotypes and one accession of *Arabis gemmifera*. In *A. thaliana*, dimorphism was also detected in the *ACL5* region, as in other nuclear genes of this plant. The nucleotide diversity (π) of the entire region, exon and intron was 0.0163, 0.0042 and 0.0293, respectively. The level of nucleotide variation in this region was among the highest of those reported for genes in this plant species. The neutrality tests of Tajima, and Fu and Li did not detect significant deviation from test assumptions for the polymorphism data. However, the HKA test indicated that the level of polymorphism in the intron was significantly high, compared with *A. gemmifera*. The high nucleotide variation in the intron is responsible for the high level of nucleotide variation in the entire region. These results can be explained by elevated mutation rate in the *ACL5* region in the *A. thaliana* lineage after the two species were split.

Key words: *ACAULIS5*, *Arabidopsis thaliana*, polymorphism, divergence

INTRODUCTION

Because plants have no nervous system, they have developed other sensing and control systems. Plants monitor a wide range of exogenous and endogenous signals and generate appropriate developmental responses to them (Gilroy and Trewavas, 2001). In plants, the hormone auxin is one of the most important endogenous signals for regulating growth and development (Gilroy and Trewavas, 2001). Auxin regulates the expression of many genes (Leyser, 2001), including *ACAULIS5* (*ACL5*), a gene in *A. thaliana* which encodes spermine synthase, a protein composed of 339 amino acids. Inactivation of *ACL5* causes a defect in the elongation of stem internodes by reducing cell expansion (Hanzawa et al., 2000). The internode elongation from the vegetative to reproductive stages is an important element in the *A. thaliana* life cycle. The number of adventitious roots formed in response to exogenous auxin differs significantly with the ecotype of *A. thaliana* (King and Stimart, 1998). Spermine synthase catalyzes the pathway that converts spermidine into spermine. Spermine is a kind of polyamine, which is involved in a wide range of processes in plants, such as triggering organogenesis and protecting against stress (Walden et al., 1997). The upstream region of the

ACL5 gene contains putative auxin-responsive cis-acting elements (Hanzawa et al., 2000).

After investigating DNA variation of thirteen loci in *A. thaliana* (Miyashita et al., 1996; Innan et al., 1996; Kawabe et al., 1997; Kawabe and Miyashita, 1999; Purugganan and Suddith, 1998; Caicedo et al., 1999; Purugganan and Suddith, 1999; Stahl et al., 1999; Kawabe et al., 2000; Kuittinen and Aguadé, 2000; Aguadé, 2001; Hauser et al., 2001; Miyashita, 2001), two unexpected features of DNA polymorphism have emerged. One is the high level of DNA variation at the species level, despite the selfing nature of this plant. The average nucleotide diversity (π : Nei and Li, 1979; Tajima and Nei, 1984) is estimated to be 0.74%. This value is higher than that of *Drosophila melanogaster* (0.4%: Moriyama and Powell, 1996). The other is a dimorphic pattern of DNA variation. In most loci, two divergent sequence types were detected. The presence of divergent sequence can account for the species polymorphism, depending on the frequency of the sequence types. The origin and maintenance mechanism of the dimorphism have been explained by three hypotheses. One is that two isolated populations of *A. thaliana* were fused before this plant species had expanded its habitat around the world (Innan et al., 1996). The second is introgression from another species. These two hypotheses do not involve natural selection. The other hypothesis is balancing selection.

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Strong support for overdominance has been obtained for *Adh* and *PgiC* (Innan et al., 1996; Kawabe et al., 2000; Miyashita, 2001). However, the exact cause and origin of the dimorphism remains to be clarified.

In this study, the level and pattern of DNA polymorphism in the 2.3-kb region of the *ACL5* locus was analyzed for *A. thaliana* and *A. gemmifera* to elucidate the genetic mechanism involved in maintaining the DNA variation in these plant species. The genes whose expressions are regulated by auxin have not been investigated yet. Therefore, *ACL5*, which, influences plant elongation, might possess DNA variations related to the phenotypic variation caused by auxin. Another purpose was to elucidate the genetic mechanism involved in maintaining the nucleotide variation in the *ACL5* region of *A. thaliana*. The study of additional regions such as the *ACL5* region might contribute to determining the cause and origin of dimorphism.

MATERIALS AND METHODS

Plant material. Twenty-one ecotypes of *A. thaliana* of various origins were used (Table 1). For interspecific comparison, one *A. gemmifera* accession sampled at Ash-

ibi, Kyoto, Japan, was used. The growth conditions for *A. thaliana* were as described previously (Kawabe et al., 2000).

DNA Extraction, PCR Amplification, and Sequencing. Genomic DNA was extracted from leaves with a modification of the CTAB procedure (Weising et al., 1991) and used for PCR amplification. A 2.3kb fragment of *ACL5* region, which is located on the short arm of chromosome 5, was amplified. PCR primers were designed based on Columbia ecotype sequence (GenBank accession AF181093). Primers for PCR amplification were 5'-ACCGAACCAAAGTAATCAAG-3', and 5'-ACGATGAGGAAGTGTGAAA-3', located in the 5' and 3' flanking regions of *ACL5*, respectively. The *ACL5* sequence of 17 ecotypes of *A. thaliana* was determined by direct sequencing of PCR products. The PCR products of the remaining three ecotypes of *A. thaliana* (Bl-1, Bus-0, Edi-0) and one accession of *A. gemmifera* (Ashibi56) were cloned into the plasmid pUC18. The plasmid DNA was used as template for the sequencing reaction. The sequencing reaction was conducted by using the Cy5 Thermo Sequenase Dye terminator kit and the Thermo Sequenase fluorescent labeled cycle sequencing kit with 7-deaza-dGTP

Table 1. Plant materials analyzed in this study

Species	Name code	Accession code	^a Origin
<i>Arabidopsis thaliana</i>	Col-0	JA58	United States
	Dra-0	JA68	Czechoslovakia
	Rou-0	JA200	Italy
	Shokei	JW101	Japan
	Ws-0	JA252	Wh,Russia
	Ag-0	JA2	France
	Nok-4	JA170	Netherlands
	Su-0	JA225	United Kingdom
	Ts-1	JA230	Spain
	In-0	JA110	Austria
	Pog-0	JA192	Canada
	Mr-0	JA155	Italy
	Hau-0	JA100	Denmark
	Ita-0	JA112	Morocco
	Bl-1	JA13	Italy
	Kas-1	JA119	India
	Edi-0	JA69	United Kingdom
	Ost-0	JA179	Sweden
	Bus-1	JA46	Norway
	Ci-0	JA54	United Kingdom
<i>Arabis gemmifera</i>	Cvi-0	JA60	Cape Verde Islands
	Ashibi56		Kyoto, Japan

^aStock number of the Sendai Arabidopsis Seed Stock Center

(Amersham/Pharmacia Biotech, Piscataway, N.J.). To eliminate PCR artifacts, we mixed the three plasmid clones at almost the same molarity. Sequencing primers were designed at ~500-bp intervals. Newly determined sequences were deposited in the DDBJ databank under accession numbers AB076723-AB076744.

Data Analysis. Including the published sequence of ecotype Columbia, a total of 21 ecotypes of *A. thaliana* were analyzed. In addition, since Edi-0 was heterozygous in the *ACL5* region and had different sequence types, each allele was treated separately and designated Edi-0 a and Edi-0 b, respectively. The region analyzed is located between sites 131 and 3021 in the coordinates of the *ACL5* sequence of the Columbia ecotype. The DnaSP program version 3.50 (Rozas and Rozas, 1999) was used to analyze intra- and inter-specific variations. Nucleotide diversity (π) and θ ($4N\mu$; Watterson, 1975) were estimated after excluding indels. Tajima's test (Tajima, 1989), Fu and Li's test (Fu and Li, 1993), MK test (McDonald and Kreitman, 1991) and HKA test (Hudson et al., 1987) were conducted to investigate the departure from neutrality. The run statistic (K_R), the Kolmogorov-

Smirnov statistics (D_{KS}), the maximum sliding G statistics (G_{max}) and the Mean sliding G statistics (G_{min}) were used to examine whether the ratio of polymorphism to divergence is uniform across a region of the DNA sequence, using the program DNA slider (McDonald, 1996, 1998).

RESULTS

The pattern of DNA polymorphism in the *ACL5* region of *A. thaliana*. A total of 155 nucleotide polymorphisms (110 sites and 45 indels) were detected, of which 139 (96 sites and 43 indels) were found more than once in the samples (Fig. 1). This frequency of non-singletons (90%; Fig. 2) is extremely high, compared to other genes studied before, where singletons are generally in excess. In the coding region, there were 17 polymorphic sites (13 synonymous sites and 4 nonsynonymous sites) and no insertions or deletions. None of the four nonsynonymous changes was non-conservative with respect to physicochemical differences, according to Miyata et al. (1979). The introns had 115 nucleotide variants (76 sites and 40 indels), of which 71 polymorphic sites and 38

Fig. 2. Frequency spectrum of polymorphic nucleotides of the entire region, exon and intron in the *ACL5* region. The expectation was calculated according to Tajima (1989).

Fig. 3. Diagram of recombination events for the six recombinant sequence types detected in this study. Each block is that shown in the figure 1. Type X is a hypothetical recombinant.

indels were non-singleton (Fig. 2). In the 5' upstream region, there were 13 polymorphic sites and 3 indel polymorphisms, but no DNA polymorphisms were detected in a 80 bp region encompassing the two putative auxin-responsive *cis*-acting elements and TATA Box. The conservativeness of this regulatory region suggests strong selective constraint on auxin-induced *ACL5* expression.

It is clear that there is dimorphism in the *ACL5* region (Fig. 1). From the distribution of the polymorphic sites, eight distinct sequence types can be defined (Fig. 1). Based on the partition pattern of ecotypes, the *ACL5* region can be divided into nine blocks. Since sequence types 1 and 8 were the most divergent, these two sequence types could be assumed to be parental. The rest could be recombinants between sequence types 1 and 8. Assuming a hypothetical recombinant sequence type X and double recombinations in the history in the *ACL5* region of *A. thaliana*, the other six sequence types could be related by seven intragenic recombinations (Fig. 3).

The times of recombination events were estimated by using the number of nucleotide differences between recombinant and parental sequence types as described for the *Adh* region of *A. thaliana* in Innan et al (1996).

Assuming that the nucleotide mutation rate per site per year is 10^{-9} as in Innan et al. (1996), we estimated that recombinant sequence types were generated 0.23–0.68 Myr ago. In contrast, the two parental sequence types were estimated to have diverged 20.92 Myr ago, the estimated times above indicates that intragenic recombinations in the *ACL5* region had occurred only very recently and in a short interval. The rapid occurrence of recombinations in *ACL5* was not observed in *Adh*, where four intragenic recombinations took place in an equal interval of about one Myr (Innan et al. 1996). Reason for the difference in recombination frequency in the two loci is not clear.

The level of nucleotide variations in the *ACL5* region. Nucleotide diversity (π) in the entire *ACL5* region was 0.016 (Table 2), which is one of the highest known among *A. thaliana* loci. Among different regions of *ACL5*, it is noted that the introns had the highest nucleotide diversity, and that the replacement variation is very low. Here, the level of variation in the introns of *ACL5* was almost twice as high as for synonymous sites in the exons (Table 2). To examine the relationship

Table 2. Summary of the intraspecific DNA variation of *ACL5* in *A. thaliana*.

	#of sites	S	π	θ	Tajima's D	Fu and Li's D*
Entire region	2341	110	0.016	0.014	0.631NS	0.976NS
Silent	1407.8	106	0.025	0.021	0.709NS	1.118NS
Coding region	1017	17	0.004	0.005	-0.991NS	-0.930NS
Synonymous	222.81	13	0.016	0.016	-0.029NS	-0.503NS
Replacement	794.19	4	0.001	0.001	-1.047NS	-1.764NS
Noncoding region	1324	93	0.024	0.022	0.808NS	1.346NS
5' flanking	256	13	0.021	0.014	1.570NS	1.088NS
Intron	976	76	0.029	0.026	0.580NS	1.317NS
3' flanking	92	4	0.019	0.012	1.446NS	1.096NS

NS: not significant

Fig. 4. Correlation of nucleotide diversity between synonymous sites in exon and intron. Regression line is for 11 gene regions except the *ACL5* and *F3H* regions. The square of the correlation coefficient is 0.450.

between levels of nucleotide variations between exon (synonymous sites) and intron, the nucleotide diversities were plotted for *ACL5* and other loci of *A. thaliana* (Fig. 4). There is no correlation between the two regions. The level of synonymous variation of *ACL5* exon was intermediate among other loci in *A. thaliana*, while the introns of *ACL5* had an exceptionally high nucleotide diversity.

χ^2 tests were conducted to examine the difference in the

number of segregating sites between the exons and introns. When all the sites were considered, the number of segregating sites in the introns (76) was significantly higher than in the exon (17, $\chi^2_{df=1} = 39.91$, $P < 0.0001$). However, no difference was detected in the number of segregating silent sites between the exon (13) and intron (76, $\chi^2_{df=1} = 0.93$, NS). Interestingly, the number of nonsingletons in the intron (71) was significantly higher than the number of synonymous sites in the

exon (5, $\chi^2_{df=1} = 7.24$, $P < 0.05$). These results indicated that the higher nucleotide diversity in the intron than in the exon (synonymous sites) is caused by the large number of nonsingletons in the intron.

Tests of neutrality on the *ACL5* region. None of the neutrality tests gave significant results for DNA variations in the *ACL5* region (Table 2). The test statistics were negative for the coding region, even for synonymous sites. This result is consistent with the observations

Fig. 5. Sliding window plot for the *ACL5* region of *A. thaliana*. (A) Nucleotide diversity of silent sites and Fu and Li's D^* for all the ecotypes are shown. Confidence intervals for 5% and 10% significance levels of a two-tailed Fu and Li's test are shown for a sample size of 22. (B) Nucleotide diversity for sequence types 1 and 8 is shown. In all cases, the window size is 100 bp, step size is 25 bp, and unit is silent site.

that the level of replacement variation was low, and that the coding region had fewer nonsingleton sites than the intron, suggesting purifying selection on the coding region. Although an extremely high level of nucleotide variation was detected in the intron, no significance in the neutrality tests was detected for the intron.

To examine the change in the level of polymorphism among functionally different regions of *ACL5*, the sliding window analysis, using Fu and Li's D^* which compares the numbers of singletons and nonsingletons, was conducted (Fig. 5-A). Positive D^* values indicate an excess of nonsingletons. There were high peaks, especially in

the introns 3, 6 and 8. Fu and Li's D^* was significantly positive around the peaks detected in the three introns, although significance was not obtained after the Bonferroni multiple correction.

A χ^2 test to examine whether the number of nonsingletons varies over different introns revealed significant heterogeneity in the number of nonsingletons among introns ($\chi^2 = 16.15$, $df = 7$ $P < 0.01$). This heterogeneity is obviously caused by the excess of nonsingletons in introns 3, 6 and 8. Figure 5-B shows sliding window plots of nucleotide variations for the two divergent sequence types 1 and 8. High peaks in the introns 3, 6 and 8 were also

Table 3. Genetic distance of nuclear genes between *A. thaliana* and *A. gemmifera*

	K	K (intron)	Ks	Ka	Ka/Ks
<i>Acl5</i>	0.034	0.109	0.147	0.006	0.038
<i>Adh</i>	0.061	0.132	0.192	0.016	0.084
<i>ChiA</i>	0.057	0.108	0.139	0.033	0.241
<i>ChiB</i>	0.054	0.297	0.157	0.025	0.156
<i>PgiC</i>	0.044	0.124	0.172	0.009	0.054
<i>Atmyb2</i>	0.073	0.119	0.233	0.033	0.141

Ks: Synonymous divergence

Ka: Replacement divergence

Table 4. Summary of HKA test on the *ACL5* and other loci

Locus		Numver of sites (bp)		Segregating sites per site		
		Within	between	Within	Between	
<i>ACL5</i> (n = 22)	Exon	1017	1017	0.017	0.033	
	Syn	222.8	222.8	0.058	0.134	
	Rep	794.2	795.2	0.005	0.006	
	Intron	836	770	0.091	0.101	
vs <i>Adh</i> (n = 17)	Exon	1137	1137	0.017	0.032	
	Syn	252.7	253	0.051	0.119	0.009NS
	Rep	884.3	884	0.009	0.016	0.488NS
	Intron	540	495	0.037	0.128	2.940NS
vs <i>ChiA</i> (n = 17)	Exon	906	906	0.049	0.054	1.027NS
	Syn	204	205.2	0.123	0.126	1.528NS
	Rep	702	700.8	0.027	0.033	0.000NS
	Intron	426	407	0.059	0.099	0.262NS
vs <i>ChiB</i> (n = 17)	Exon	1008	1008	0.022	0.052	0.023NS
	Syn	240.3	240.3	0.071	0.141	0.098NS
	Rep	767.7	767.7	0.005	0.024	1.947NS
	Intron	427	190	0.056	0.245	6.069*
vs <i>PgiC</i> (n = 21)	Exon	1667	1656	0.018	0.042	0.037NS
	Syn.	388.2	383.6	0.041	0.153	0.560NS
	Rep	1288.8	1269.4	0.012	0.010	0.068NS
	Intron	2837	2823	0.032	0.114	6.609*

* $P < 0.05$; NS: not significant

detected. When the sequence types 1 and 8 were considered separately, the peaks disappeared. This result indicates that these peaks in introns are caused by difference between the divergent sequence types.

The divergence in the *ACL5* region between *A. thaliana* and *A. gemmifera*. *ACL5* has the lowest divergence between *A. thaliana* and *A. gemmifera* in both the coding and intron regions among all the genes compared (Table 3). Corresponding with the low replacement polymorphism (Table 2), replacement divergence of *ACL5* was also the lowest, suggesting again purifying selection on the amino acid substitutions in the coding region of *ACL5*.

HKA tests were applied to examine the correlation between polymorphism in *A. thaliana* and divergence between the two species (Table 4). No significant deviations were detected for the exons. On the other hand, the HKA test for intron indicated that *ACL5* was significantly different in comparison with *ChiB* or *PgiC*, and revealed a marginally significant difference compared with *Adh*. In these cases of HKA test, obviously the high level of polymorphism in intron of *ACL5* contributed to the significance (and nearly significance), although signif-

icance was not obtained after the Bonferroni multiple correction. It can be concluded that the *ACL5* intron has a significantly higher ratio of polymorphism and divergence compared to those of introns of other loci in *A. thaliana*. The MK tests found no significant results.

Figure 6 shows a sliding window plot for silent divergence between the two species. High peaks of divergence in the three introns (3, 6, and 8) were detected, as for polymorphism. The tests of heterogeneity in the ratio of silent polymorphism to divergence (McDonald, 1996, 1998) were applied to the *ACL5* region, but they did not reveal any significance. It can be concluded that the distribution of divergence was compatible with that of polymorphism. When sequence types 1 and 8 were analyzed separately, high peaks of divergence in the introns, especially intron 3, were still found (data not shown). Therefore, it can be concluded that the peaks of divergence in the introns were not due to the dimorphic variation.

The 5' upstream region containing putative auxin-responsive cis-acting elements and TATA box is conserved between *A. thaliana* and *A. gemmifera*. Although 18 substitutions were detected (7.2%) in the entire 5' region, in the 80 bp region with the two regulatory ele-

Fig. 6. Sliding window plot for the *ACL5* region between *A. thaliana* and *A. gemmifera*. Open boxes indicate exons, and the black box indicates the putative auxin-responsive cis-elements (AuxREs). Window size is 50 bp, step size is 10 bp.

ments and TATA Box, only one site substitution was detected. Around the region of auxin regulation, no peak of variation was detected in either polymorphism or divergence (Fig. 6). These results suggest that regulation of auxin-induced *ACL5* expression is essential for both species.

DISCUSSION

DNA polymorphism in the *ACL5* region of *A. thaliana*. As in some other nuclear loci of *A. thaliana* (11 out of 13 loci studied so far), the *ACL5* region was found to be dimorphic. Intragenic recombinations in the *ACL5* region occurred more recently and in a shorter interval than those in the *Adh* region. Although there may be some genetic mechanisms underlying this difference, a possible explanation would be subpopulation structure in the ancient *A. thaliana* population before spreading over the world. Supposing that the ancient population consisted of many isolated subpopulations, differentiation of divergent sequence types in each of the two regions would proceed independently in different isolated populations. Fused populations could be segregating in *Adh* but not in *ACL5*, and *vice versa*. Probably, fusion of isolated populations containing different sequence types occurred only recently for *ACL5*. The exact reason for the difference in intragenic recombination in the two regions is not clear.

This study demonstrated that the nucleotide variation in the *ACL5* region is one of the highest among *A. thaliana* nuclear genes, which is due to the unusually high level of variation in the introns. Also, in this region the number of nonsingletons was very high, unlike other regions studied in this species. Especially the introns had significantly more nonsingletons than the coding region (synonymous sites). The HKA test statistically confirmed that the level of nucleotide polymorphism in introns of *ACL5* was high.

Despite the high level of variation in the entire region, the 5' flanking region containing putative auxin-responsive cis-acting elements and TATA box is highly conserved, even in the interspecific comparison with *A. gemmifera*. This result indicated that auxin regulation in *ACL5* is essential in these two species. To clarify if the low level of variation in the auxin-regulatory region is gene- or genus-specific, DNA variation in other auxin-regulated genes must be analyzed in the future.

High level of nucleotide variation in the introns of *ACL5* region. One possible explanation for high level of polymorphism is a high mutation rate. However, divergence between *A. thaliana* and *A. gemmifera* was not unusually high compared with other loci. If the mutation rate is related to the high level of polymorphism, the mutation rate in this region must have been elevated in the *A. thaliana* lineage after split of the two species. The observation that the coding region of *ACL5* had the same

levels of polymorphism as those of other loci could be explained by purifying selection. Since introns could be subjected to less stringent selection pressure than the coding region, a high mutation rate would be reflected directly on the high level of the variation in the introns. Coincidentally, the *ChiA* locus located on the short arm of chromosome 5, as the *ACL5* locus, also has a high level of nucleotide variation (Kawabe et al., 1997). Regional hotspots of nucleotide mutation might exist in the *A. thaliana* genome.

The other possibility for a high level of polymorphism is a balancing selection. There are high peaks of nucleotide variation in three introns, which disappear when the two divergent sequence types were analyzed separately. This kind of pattern of DNA variation was invoked as an evidence of balancing selection, such as overdominance, for *Adh* of *Drosophila melanogaster* (Hudson and Kaplan, 1988), and *Adh* and *PgiC* of *A. thaliana* (Innan et al., 1996; Kawabe et al., 2000; Miyashita, 2001). In these previous cases, peaks of nucleotide variation were clearly associated with replacement sites in the exon region, which are responsible for allozyme variation of these genes. Here, it seems difficult to assume natural selection, since the peaks were found in introns, which do not influence protein product. However, it has been shown that DNA variation in intron influences the enzyme activity of ADH in *Drosophila melanogaster* (Laurie et al., 1991), where an indel polymorphism detected in the first intron is related to a change in enzyme concentration, affecting the activity. In intron 3 of *ACL5*, where the highest peak of nucleotide variation was detected, a duplicated sequence was found polymorphic between the two divergent sequence types. This indel might be related to *ACL5* expression, as the indel in *Adh* of *D. melanogaster*. At this moment, it is not possible to specify the molecular basis of balancing selection for *ACL5*. Further molecular analyses on enzyme activity and gene expression are necessary to specify if the high peaks of nucleotide variation in introns are related to some form of natural selection or not.

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