A mutant of Arabidopsis with reduced internodal cell length, acaulis (acl), has recently been shown to have reduced transcript levels of a gene for endoxyloglucan transferase, EXGT-A1 (Y. Hanzawa, T. Takahashi, Y. Komeda [1997] Plant J 12: 863–874). In the present study, we cloned genomic fragments of five members of the EXGT gene family, EXGT-A1, EXGT-A3, EXGT-A4, XTR2, and XTR3, and examined their expression in the wild type and in a series of acl mutants. In wild-type plants, the EXGT-A3 gene showed higher expression in lower internodes (internodes between nodes bearing axillary shoots) than in upper and young internodes, in which EXGT-A1 was highly expressed. EXGT-A4 was preferentially expressed in roots and XTR3 in siliques. The XTR2 gene was constitutively expressed. In acl1, acl3, and acl4 mutants, which have a severe defect in leaf expansion as well as in internode elongation, the EXGT-A1 gene showed reduced levels of expression before bolting of plants. In contrast, XTR3 was increased in these mutant seedlings. Reduction of EXGT-A1 expression was also detected after bolting of all acl mutants except acl2, whose growth defect is restricted to lower internodes. These results suggest the involvement of each EXGT in different aspects of organ development.

The growth of plant cells depends on the balance between the turgor pressure and the extensibility of the cell wall. While the turgor pressure, which provides the driving force for cell extension, is influenced by the availability of water, the wall extensibility is to a large extent regulated by enzymes involved in the cleavage or formation of cross-links between cell wall polymers and in the turnover of certain wall components. In dicots, xyloglucan is a major structural polysaccharide of primary cell walls and is hydrogen-bonded to cellulose microfibrils to form cross-links between them (for review, see Hayashi, 1989; Carpita and Gibeaut, 1993). The cleavage and molecular grafting of xyloglucan polymers are catalyzed by endoxyloglucan transferase (EXGT) enzymes (also called xyloglucan endotransglycosylase; XET). Therefore, EXGT has been suggested as one of the most likely agents responsible for wall loosening (for review, see Fry, 1995; Nishitani, 1995, 1997).

Cloning of EXGT genes from several plant species has led us to realize that plants possess a large gene family of EXGTs. They have been classified into three subfamilies based on their sequence similarities (Nishitani, 1995, 1997; Xu et al., 1996). Subfamily I includes EXGT-V1 from azuki bean epicotyls, the first enzyme proved to mediate a transglycosylation reaction between xyloglucans (Nishitani and Tominaga, 1992). Subfamily II includes Arabidopsis meristem-expressed Meri5 (Medford et al., 1991) and mechinostimulus-inducible TCH4 (Xu et al., 1995), soybean brassinosteroid-inducible BRU1 (Zurek and Clouse, 1994), maize flooding-responsive WUSL1005 (Saab and Sachs, 1996), and tomato-fruit-expressed XET-B1 (Arrowmith and de Silva, 1995). Germinating seed-specific NXG1 of nasturtium (de Silva et al., 1993) belongs to subfamily III and was originally identified as a hydrolase (xyloglucanase) (Edwards et al., 1986). Expression patterns of these genes are in good agreement with their proposed roles in cell wall modification during cell elongation (Nishitani, 1997), fruit ripening (Redgwell and Fry, 1993), vascular differentiation (Oh et al., 1998), and adaptive growth to physical stimuli (Antosiewicz et al., 1997). Dwarf phenotypes of the Arabidopsis brassinosteroid-responsive mutants have been shown to correlate with a reduced expression of TCH4 (Kauschmann et al., 1996). It is still not known whether each member of the EXGT gene family within a single plant species plays a distinct and vital role in cell morphogenesis.

We have isolated and studied Arabidopsis mutants with reduced internodal cell length, acaulis (acl), to determine the molecular basis of cell elongation in stem internodes. In rosette plants, including Arabidopsis, initiation of the internode elongation (bolting) follows flower bud formation. This process is probably mediated by phytohormones, but how their effects are exerted is not clear. Our previous study revealed that the acl5 mutant, whose defect is sharply restricted to internodal growth, shows a reduced expression of the EXGT-A1 gene after flowering (Hanzawa et al., 1997). We report the cloning of genomic fragments of five members of the EXGT gene family and their expression patterns in the wild type and in a series of acl mutants, to which acl3 and acl4 have recently been added.

Expression of Endoxyloglucan Transferase Genes in acaulis Mutants of Arabidopsis

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1 This work was partially supported by a Grant-In-Aid from the Ministry of Education, Science and Culture of Japan and by a grant for the Research for the Future Program from the Japan Society for the Promotion of Science (JSPSRTFP96L00403).

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MATERIALS AND METHODS

Plant Material

Arabidopsis ecotype Columbia was used in all experiments. Plants were grown on rock-wool bricks watered with Murashige and Skoog solution under continuous fluorescent light at 22°C. For RNA preparation from root tissue, seeds were surface-sterilized and sown on solidified Murashige and Skoog medium with 3% (w/v) Suc in Petri dishes. Petri dishes were kept under continuous fluorescent light at 22°C.

The acl3-1 and acl4-1 mutants were selected in a screen for mutants with short internodes from M2 plants derived from fast-neutron-mutagenized seeds homozygous for gl1 (Lehle Seeds, Tucson, AZ). These were backcrossed five times into the wild-type Columbia (Col-0). Mapping was performed using molecular markers polymorphic between Columbia and Landsberg erecta (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). Mutant alleles of ACL1 and ACL2 used in this study were acl-2 and acl-1, respectively (Tsukaya et al., 1993). The acl5-1 allele in the Landsberg erecta background (Hanzawa et al., 1997) was backcrossed at least five times into the Columbia background.

Isolation of Genomic Clones Encoding EXGT

Four cDNA clones with homology to EXGT-A1 (Okazawa et al., 1993) were previously isolated from an Arabidopsis cDNA library by screening at low stringency with the EXGT-A1 cDNA fragment, and were designated EXGT-A2, EXGT-A3, EXGT-A4, and EXGT-A5 (Nishitani, 1997; S. Okamoto and K. Nishitani, unpublished data). The nucleotide sequences of EXGT-A2 and EXGT-A5 were found to be identical to those isolated and named XTR2 and XTR3, respectively, by Xu et al. (1996). An Arabidopsis genomic library constructed in AGEM12 was generously provided by J. Mulligan and R.W. Davis (Stanford University, Stanford, CA). The library was screened by plaque hybridization using a mixture of cDNA fragments as the probes. Subclones were prepared in pBluescript SK+ (Stratagene, La Jolla, CA) and sequenced using a Taq dye terminator cycle sequencing kit and a DNA sequencer (model 373A, Applied Biosystems, Foster City, CA).

RNA Gel-Blot Analysis

Total RNA was isolated from different tissues as described by Takahashi et al. (1992), separated by agarose/formaldehyde gel electrophoresis, and blotted onto nylon membranes (GeneScreen, New England Nuclear, Boston). Hybridization was performed at 42°C in 50% (w/v) formamide, 10% (w/v) dextran sulfate, 1% (w/v) SDS, 1 M NaCl, 0.25 mg mL⁻¹ salmon-sperm DNA, and the labeled gene-specific probe (see below). The filters were washed twice for 15 min at 65°C in 2× SSC, 1% (w/v) SDS and once at room temperature in 0.1× SSC. For all blots, equal loading was confirmed by ethidium bromide staining of ribosomal RNAs (25S, 18S).

Figure 1. Comparison of EXGT genes. A, Genomic structure of the EXGT genes cloned in this study. Protein coding regions are shown by black boxes with the number of amino acid residues encoded by each exon. Numbers in parentheses indicate the number of nucleotides for intron. Intron splice sites in genomic sequences were deduced by comparison with their corresponding cDNA sequences, EXGT-A1 (Okazawa et al., 1993; accession no. D16454), XTR2 (Xu et al., 1996; accession no. U43487), EXGT-A3 (Nishitani, 1997; accession no. D63509), EXGT-A4 (Nishitani, 1997; accession no. AB026486), and XTR3 (Xu et al., 1996; accession no. U43485). The accession numbers for genomic sequences determined in this study are AF163819 (EXGT-A1), AF163820 (XTR2), AF163821 (EXGT-A3), AF163822 (EXGT-A4), and AF163823 (XTR3), respectively. B, Phylogenetic relationship between the Arabidopsis and other EXGT-related protein sequences. The entire deduced amino acid sequences were compared using the malign program of DNA Data Bank of Japan (Nishitani, 1997). References: a, Arrowsmith and de Silva (1995); b, Xu et al. (1995); c, Xu et al. (1996); d, Medford et al. (1991); e, Saab and Sachs (1995); f, Zurek and Clouse (1994); g, Nishitani (1997); h, Okazawa et al. (1993); i, Rose et al. (1996); and j, de Silva et al. (1993).
Probe Preparation

To specifically detect each of the EXGT transcripts in the RNA gel-blot hybridization, 3'-end-specific probes were synthesized by PCR using cDNA clones as templates. The PCR primers were A1F (5'-GGCGGTTTAGAGAAAGACCAA-3'), A1R (5'-GTAACCTAGGGCTCTGTC-3'), A2F (5'-AAGCCGTCTCAGGTCTATGA-3'), A2R (5'-GTTCATAAAATGGAGGAAATC-3'), A3F (5'-CAGTTTCCGAGCTGATGA-3'), A3R (5'-GGCCAAATCTCACCACTACTG-3'), A4F (5'-TGCACTGACACGTT-3'), A4R (5'-CCAACCTCTAGATTAAATTGA-3'), A5F (5'-TAGCTAGAATTAATGTG-3'), and A5R (5'-AACCAACATACATCCC-3'). The specificity of each probe was confirmed by DNA gel-blot analysis. No cross-hybridization was observed (data not shown). The PCR products were agarose gel purified and labeled by the random-primer protocol (BcaBest Labeling Kit, Takara, Kyoto).

RESULTS

Genomic Structure of EXGT Genes

Five EXGT cDNA clones (Okazawa et al., 1993; Nishitani, 1997; S. Okamoto and K. Nishitani, unpublished data) were used as probes to screen an Arabidopsis genomic library in λGEM12. Sequence analysis of subcloned genomic DNA fragments revealed the presence of two or three introns whose placement within each of the EXGT coding regions is conserved (Fig. 1A). The phylogenetic tree for these genes and those identified from other plant species is shown in Figure 1B. Genomic DNA-blot analysis indicated that 3'-end-specific probes prepared from these EXGT genes (see “Materials and Methods”) hybridized to a single-copy gene at high-stringency conditions (data not shown).

Developmental Regulation of EXGT Gene Expression

Steady-state levels of EXGT transcripts were measured in different organs of adult flowering plants and in young seedlings before bolting. The results of RNA-blot hybridization using 3'-end-specific probes are shown in Figure 2. The EXGT-A1 gene was highly expressed in 7-d-old seedlings and in the roots, upper internodes (internodes between nodes bearing flowers), flower buds, and green siliques of 30-d-old flowering plants. Transcript levels in fully expanded leaves and lower internodes (internodes between nodes bearing axillary shoots) were reduced, indicating the preferential expression of the EXGT-A1 gene in young, developing tissues. On the other hand, XTR2 showed a constitutive expression. EXGT-A3 showed a pattern similar to that of XTR2, but was higher in lower internodes. The EXGT-A4 gene was mainly expressed in roots. XTR3 was restricted to siliques and only weakly expressed in mature leaves. We further examined the expression of EXGT-A1 and EXGT-A3 genes during the internode elongation. RNA samples were prepared from upper and lower internodes at 5, 10, and 15 d after bolting, respectively. Our results revealed that, while the EXGT-A3 expression was increased as the day proceeded, the EXGT-A1 expression, especially in lower internodes, was drastically decreased (Fig. 3).

Identification of New acl Loci

The acl mutants have been characterized by a defect in elongation growth of stem internodes after flowering, from which the name “acaulis” originates. In addition to the
previously described mutants acl1, acl2, and acl5, two mutants derived from fast-neutron-mutagenized plants were found to represent new recessive loci by complementation tests and defined as acl3 and acl4, respectively (Fig. 4). Mapping experiments revealed that acl3 is tightly linked to the marker GL1 (Konieczny and Ausubel, 1993) on chromosome III and that acl4 is tightly linked to the marker SC5 on the lower arm of chromosome IV (data not shown). These two mutants have a severe defect in rosette leaf expansion before flowering and are phenotypically indistinguishable from the allele of acl1-2 (Fig. 5A).

We found that, like the phenotype of acl1 (Tsukaya et al., 1993), the phenotype of acl3 and acl4 could not be rescued by the exogenous addition of phytohormones, but was drastically suppressed by elevated growth temperature (28°C; Fig. 5B). On the other hand, acl2 and acl5 mutants were nearly wild-type in appearance before bolting and their defect was only detected in the growth of stem internodes (Fig. 4). In contrast to acl1, acl3, and acl4 mutants, whose internodal growth was markedly restored at 28°C, acl2 and acl5 mutants showed no restoration of the internodal growth at 28°C. The reduction in leaf expansion and/or stem elongation in all of these acl mutants is primarily due to the reduction in cell size (Tsukaya et al., 1993; Hanzawa et al., 1997; data not shown).

EXGT Gene Expression in acl Mutants

The effect of acl mutations on the expression of EXGT genes was examined by RNA blots. Figure 6A shows that the EXGT-A1 expression was reduced in aerial portions of 7-d-old seedlings of acl1, acl3, and acl4 mutants with the leaf phenotype. Interestingly, these three mutant seedlings exhibited elevated levels of the XTR3 transcript. Reduced expression of EXGT-A1 was also observed in acl5 mutants after flowering, as well as in acl1, acl3, and acl4 flowering plants (Fig. 6B). The transcript levels of XTR3 in 30-d-old flowering plants, which seems mainly attributable to the

Figure 4. Morphology of adult flowering plants with acl mutations. Plants were grown at 22°C under continuous light for 40 d. A, acl1-2; B, acl2-1; C, acl3-1; D, acl4-1; E, acl5-1. Scale bars = 1 cm.
expression in siliques (Fig. 2), and those in rosette leaves of flowering plants were unaffected by these acl mutations (Fig. 6B; data not shown). There were no obvious influences of acl mutations on the transcript levels of XTR2 and EXGT-A3 in aerial tissues (Fig. 6, A and B) or those of EXGT-A1 and EXGT-A4 in roots (Fig. 6C). We further found that the transcript levels of EXGT-A1 in acl1, acl3, and acl4 seedlings grown at 22°C were restored by the growth at 28°C, in parallel with their morphological phenotypes (Fig. 6D). An elevated level of EXGT-A1 expression was also seen in wild-type seedlings grown at 28°C, in which leaf expansion and petiole elongation were also enhanced (Fig. 5).

DISCUSSION

One of our major interests was to identify actual molecules involved in the rapid cell growth of stem internodes in Arabidopsis. Previously, we observed that the acaulis5 (acl5) mutant showed a marked reduction of the EXGT-A1 gene expression after flowering, as well as a severely reduced length of stem internodes (Hanzawa et al., 1997). To evaluate the relationship between the expression of EXGT genes and plant cell growth, we extended our analysis to the expression of other members of the EXGT gene family in the wild type and in a series of acl mutants.

This study revealed that the members of the EXGT gene family are under the differential control of expression during development of wild-type plants. Expression of EXGT-A3 appeared to be high in lower (old) internodes, in contrast to that of EXGT-A1 in upper (young) internodes. According to the phylogenetic tree established from related protein sequences (Fig. 1B; Nishitani, 1997), EXGT-A1 and root-expressed EXGT-A4 belong to subfamily I, while XTR2 and EXGT-A3 belong to subfamily III. In nasturtium, NXG1 (subfamily III) and XET1 (subfamily I) exhibit mutually exclusive patterns of gene expression and possess different substrate specificities (Rose et al., 1996). NXG1 has been suggested to act predominantly as a hydrolytic enzyme in the mobilization of xyloglucan seed storage reserves in germinating seed cotyledons (Edwards et al., 1986). If hydrolytic action toward xyloglucans is a major role of members of subfamily III, then EXGT-A3, together with XTR2, could be required for the regulated degradation of xyloglucan networks for the maturation and/or maintenance of the fine structure of cell walls, which follows the elongation growth. It will be necessary to determine whether these EXGTs possess different enzyme activities against different xyloglucan substrates and whether they exhibit cell-type-specific patterns of expression.

The significance of EXGT-A1 in cell elongation was strengthened by our analysis of the expression in acl mutants. Two loci, acl3 and acl4, were newly identified in this study. The phenotypes of these two mutants could not be restored by exogenously applied phytohormones (data not shown), suggesting that neither of these mutations represent genes involved in hormone biosynthesis. Based on their phenotypes, which are almost identical to the acl1 phenotype, we suggest that these three gene products act in a common regulatory pathway of cell elongation.

Our results showed that the defects of acl1, acl3, and acl4 in leaf expansion and in stem elongation are accompanied by
There were no detectable alterations in EXGT-A1 expression in semidominant acl2 mutants. This can be explained by the limited defect of acl2 within the internode elongation between nodes bearing axillary shoots (Tsukaya et al., 1995), which might be accompanied by a temporal and slight reduction in EXGT-A1 expression. However, it is also likely that the acl2 mutation has a negative effect on other molecules involved in cell elongation, while having no influence on EXGT-A1.

Preferential expression of the XTR3 gene in wild-type siliques is consistent with the fact that the corresponding cDNAs have been identified as expressed-sequence-tag clones derived from dry seeds by Xu et al. (1996). XTR3, as well as stress-responsive Meri5 and TCH4 (Xu et al., 1996), belongs to subfamily II. We found that, in contrast to EXGT-A1, the XTR3 transcript levels were elevated in acl1, acl3, and acl4 seedlings. Such opposite effects on EXGT genes may reflect the complexity of environmental and hormonal regulation of the EXGT gene expression (Xu et al., 1996). Cloning of the ACL genes is currently in progress and will help to answer the question of how acl mutations affect regulatory pathways of EXGT gene expression.

In summary, our data on the expression of EXGT genes (especially on their responsiveness to environmental stimuli), which are supported by data reported by others, support the possibility that many kinds of mutations can affect the regulatory pathways of EXGT gene expression, resulting in altered cell morphology. The molecular processes underlying the cell wall architecture consist of various biochemical steps, indicating the involvement of many enzymes other than EXGT. It should be noted that there is increasing evidence suggesting the importance of expansins (Cosgrove, 1998) and endo-1,4-β-glucanases (Shani et al., 1997; Nicol et al., 1998) in plant cell growth. Expansins have been identified as a catalyst for acid growth and have been shown to induce the extension of isolated cell walls (McQueen-Mason and Cosgrove, 1995).

**ACKNOWLEDGMENTS**

We are grateful to Drs. John Mulligan and Ronald W. Davis (Stanford University, Stanford, CA) for the gift of the Arabidopsis genomic library. We also thank Dr. Shigehisa Okamoto (Kagoshima University, Kagoshima, Japan) for help with the cloning of EXGT genes.

Received April 21, 1999; accepted July 8, 1999.

**LITERATURE CITED**


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