The *ATE* Genes Are Responsible for Repression of Transdifferentiation into Xylem Cells in Arabidopsis[1]

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We isolated three recessive mutants of Arabidopsis (*Arabidopsis thaliana*) showing ectopic expression of the xylem-specific marker, *pAtxyn3*::*YFP*. Genetic analysis indicated that the phenotypes were caused by mutations in three different genes, designated *Abnormal Tracheary Element formation-related gene expression* (*ate1–3*). The *ate1* mutants showed a normal *DR5*::*GUS* gene expression pattern, and the *ate1* mutation did not affect the abnormal vascular pattern formation in the *van3* and *pin1* mutants, indicating that the *ate1* mutation does not affect the vascular pattern organization governed by auxin. The *ate* mutants showed ectopic lignin deposition, patterned secondary wall thickenings, and cell death, which are characteristic of mature tracheary elements (TEs) in cells ectopically expressing the *pAtxyn3*::*YFP* gene. Ectopic TE formation was rapidly induced in parenchymal tissue of the *ate* mutants in a TE-inducible system with excised hypocotyl. Furthermore, reverse transcription-polymerase chain reaction experiments showed that the expression of TE formation-related genes is up-regulated in the *ate* mutants. The *ate1* mutation also caused ectopic expression of another xylem-specific marker gene, *pAt3g62160*::*YFP*. Overall, our results suggest that the *ATE* genes are responsible for the in situ repression of transdifferentiation into TEs in Arabidopsis and could be participants in the transdifferentiation-masking system.

In contrast to animals, plants have a flexible transdifferentiation ability. Many differentiated plant organs, tissues, and cells retain the ability to regenerate all parts of the plant body. Plants can regenerate in tissue culture, as demonstrated by Stewart (1958), who observed somatic embryogenesis in carrot (*Daucus carota*). The obvious conclusion from these experiments is that even single vegetative carrot cells retain their totipotency. In organogenesis, plant organs can be produced without embryonic development. Various individual organs such as roots, shoots, flower buds, and inflorescences can be regenerated, in most cases, via callus from somatic tissues or somatic cells (Lu, 2003).

Why is it that transdifferentiation is not expressed during normal plant development? Why can many types of organs (cells) be transdifferentiated from somatic cells? These basic questions may be explained by the transdifferentiation-masking system that exists in normal plant development enabling rapid reorganization of the plant body, depending on external stimulus. In fact, some genes prevent transdifferentiation, such as *PICKLE* (*PKL*), a gene identified in Arabidopsis (*Arabidopsis thaliana*) that prevents roots from making embryos. The roots of *pkl* mutants express embryo-specific genes. If *pkl* roots are placed in culture medium without hormones, they will occasionally generate somatic embryos, unlike roots from wild-type plants, which require hormones. Furthermore, overexpression of the *LEAFY COTYLEDON* gene induces ectopic embryo formation in vegetative tissues such as cotyledons, and the *PKL* gene is required to repress *LEAFY COTYLEDON* (Wilt and Hake, 2004).

In the transdifferentiation of somatic cells, reorganization of the gene expression pattern, the methylation pattern of genomic DNA, histone acetylation, telomere elongation, and some other events is required. These events can be modified depending on the transdifferentiating cell (organ) types. Despite the importance of the transdifferentiation system in plant biology, very little is known about the underlying molecular mechanisms.

The first hints about how plants regulate transdifferentiation came from studies on carrot cell cultures (Reghavan, 1986). Pretreatment with a synthetic auxin is essential for inducing the embryogenic potential of callus cells, and the subsequent removal of auxin leads to the formation of somatic embryos (Stewart et al., 1958; Reinhert, 1959). Auxin seems to be one of the key regulators in dedifferentiation and subsequent somatic embryogenesis. However, it is still unknown how auxin regulates transdifferentiation.

Restricted model systems have clarified the difficulties of analyzing the molecular mechanisms of the

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transdifferentiation system. Dedifferentiation induced by nuclear transplantation, stress, hormone treatment, and other factors, and subsequent callus formation or somatic organogenesis are usually accompanied by many types of cell differentiation, including various types of molecular events such as stress responses, hormone responses, healing, and cell division. Thus, it is difficult to focus on the mechanisms of the transdifferentiation system. In this situation, tracheary element (TE) transdifferentiation is an excellent study example occurring at the cellular level in vascular plants (Fukuda, 1994). TE transdifferentiation may be one of the best indicators for analysis of the molecular mechanisms underlying the plasticity of differentiation potency in plants because the molecular events of single cell transdifferentiation are less complex in comparison with that of organ (tissue) regeneration, which comprises many different types of cell differentiations. Furthermore, TEs are easily identified because of their characteristic pattern of secondary wall thickening and subsequent cell death. By using the simple in vitro TE transdifferentiation system of zinnia (*Zinnia elegans*), in which isolated mesophyll cells transdifferentiate into TEs, various aspects of the TE development have been revealed (Fukuda, 2004). Nevertheless, it has been difficult to study the transdifferentiation-masking mechanism that occurs in situ using the in vitro zinnia system because the in vitro transdifferentiation processes include various responses to artificial external stimuli such as excision stress, culture stress, and supplied plant hormones.

As genetic analysis of the TE transdifferentiation in situ seems to be the best approach to overcoming such difficulties, we isolated mutants showing ectopic TE transdifferentiation. For effective screening of this type of mutant, we used a transgenic plant harboring a TE-specific marker. We have previously demonstrated the TE-specific expression of the xylanase gene of *Z. elegans*, *Z6874* (Demura et al., 2002). Here we showed that its Arabidopsis homolog, *Atxylanase3*, is expressed in a TE-specific manner. Using transgenic plants harboring *Atxyn3*::*YFP*, we isolated Arabidopsis *Abnormal Tracheary Element formation-related gene expression 1* to *3* (*ate1–3*) mutants, which exhibited ectopic expression of the TE marker, *Atxyn3*::*YFP*. From physiological, genetic, and molecular analyses, we suggest that the *ATE* genes are responsible for the repression of transdifferentiation into TEs in situ, and that the *ATE* genes may participate in the transdifferentiation-masking system.

RESULTS

Construction of a Xylem Cell Marker Line

We have shown previously that xylanase gene (*Z6874*) expression is detected only in developing TEs in *Z. elegans* (Demura et al., 2002). Arabidopsis *Atxylanase 3* (*Atxyn3*::*At4g08160*) exhibits significant sequence homology to *Z6874* (data not shown). To test the possibility of using the *Atxyn3* gene promoter as a TE molecular marker, we analyzed the *Atxyn3* promoter region 2 kb upstream of the start codon, using a *yellow fluorescence protein* (*YFP*) reporter gene in transgenic Arabidopsis. Four independent transgenic plants showed the same marker gene expression pattern, and we selected one transgenic plant, *Atxyn3*::*YFP*, as a xylem marker line. In the root, xylem cell maturation starts in the maturation zone (Fig. 1A), and *YFP* gene expression was observed in maturing TEs in the root maturation zone (Fig. 1, A and B). In our system, a nuclear localization signal was translationally fused with YFP, and the YFP signal was observed only in the nucleus of the developing TEs (Fig. 1, B and D). The YFP signal appeared transiently. As shown in Figure 1, B and C, the YFP signal appeared just before, and disappeared just after, the vacuole collapsed. Differentiation of TEs in the cotyledon varies greatly with developmental stage. TE development is repressed during embryogenesis and occurs at 2 d after germination (DAG; Busse and Evert, 1999a, 1999b). After 3 DAG, only the differentiating TEs show the YFP signal. Figures 1D, 2E, and 5C show the different developing stages of cotyledons, at 2 DAG, 3 DAG, and 14 DAG, respectively. At 2 DAG, most TEs are developing and show the YFP signal (Fig.

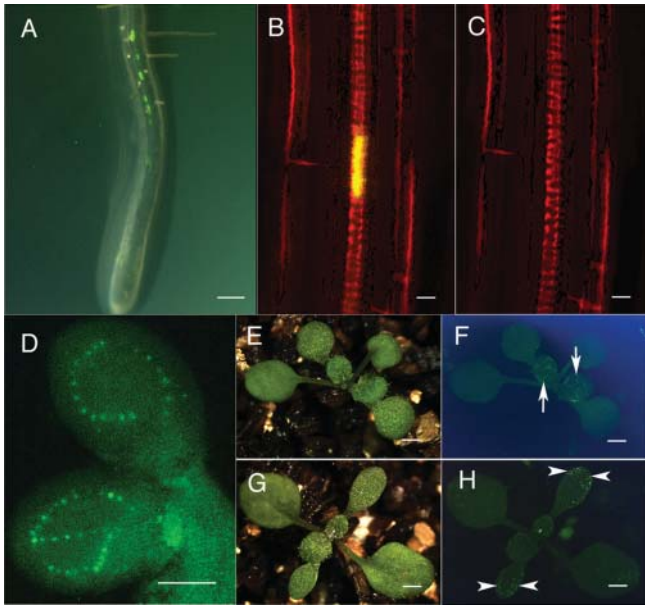

Figure 1. *Atxyn3*::*YFP* marker gene expression. Wild type (A–F) and *ate3* mutant (G and H) are shown. YFP signals were examined by fluorescent microscope (A, D, F, and H) and by a confocal laser scanning microscope (B and C). E and G are daylight images. In the wild-type seedlings, YFP fluorescence signals appeared along a line of the vasculatures of the root (A) and cotyledon (D and F). The YFP signal is detected in the nucleus of a developing TE (B), but it is lost during maturation of a TE (C). In the *ate* mutant, ectopic YFP signals appear independent of vasculatures (H). White arrows in F show YFP signals in the developing TEs. White arrowheads in H show ectopic YFP signals. Scale bars, 100 μm (A), 10 μm (B and C), and 1 mm (D–H).

1D). At 14 DAG, most TEs are already mature and show no YFP signal (Fig. 5C). YFP signals were observed only in the developing TEs of all organs tested, i.e. the roots, hypocotyls, cotyledon, leaf, sepal, petal, stamen, and carpel. TE differentiation is accompanied by programmed cell death, and senescence, wound, and pathogen responses also involve programmed cell death. However, the *Atxyn3*::*YFP* marker gene was not expressed in wounded tissues or tissues undergoing senescence (data not shown). Tao et al. (2003) performed a comprehensive analysis using the Affymetrix GeneChip to study Arabidopsis responses to a bacterial pathogen and found the *Atxyn3* expression was not induced by the pathogen. These results indicate that *Atxyn3*::*YFP* can be used as a developing TE-specific marker.

Isolation of Mutants Showing Abnormal Xylem Marker Gene Expression

The cotyledon of Arabidopsis has a very simple vein pattern, i.e. one midvein and three or four lateral veins (Fig. 1D). Using this pattern as an index, we examined mutagenized *Atxyn3*::*YFP* transgenic lines for mutants showing the ectopic *YFP* expression pattern, in order to investigate the molecular mechanisms of the transdifferentiation repression system. The screening of 10,000 M_2 plants resulted in the isolation of 3 mutants with ectopic marker gene expression (Fig. 1, E–H). In each mutant line, we observed segregation of the seedlings that exhibited aberrancy in the *YFP* expression pattern in a ratio (3:1) consistent with a single recessive lesion. In the complementation test, by crossing the three mutants with each other, it was found that these three mutants represented single mutant alleles at three genetic loci. These mutants were designated as *ate1*, *ate2*, and *ate3*. We mapped these *ATE* loci to chromosome 1 between nga280 and nga111 (*ATE1*), to chromosome 5 between nga151 and nga139 (*ATE2*), and to chromosome 3, the northern part of nga126 (*ATE3*).

Vascular cell maturation starts 2 or 3 DAG (Busse and Evert, 1999a, 1999b), and the *YFP* signal in *Atxyn3*::*YFP* transgenic plants was also evident at 3 DAG in Arabidopsis (Fig. 2, A and E). Ectopic expression was observed from 10, 10, and 3 DAG in the *ate1*, *ate2*, and *ate3* mutants, respectively (Fig. 2, B–D, F–H, and L–N). In the *ate1* mutants, patches of *YFP*-expressing cells were observed (Fig. 2L). YFP signals were present randomly in the parenchyma tissues of the *ate2* and *ate3* mutants (Fig. 2, H, M, and N). The *ate1* and *ate2* mutants produced pale green organs (Fig. 2,

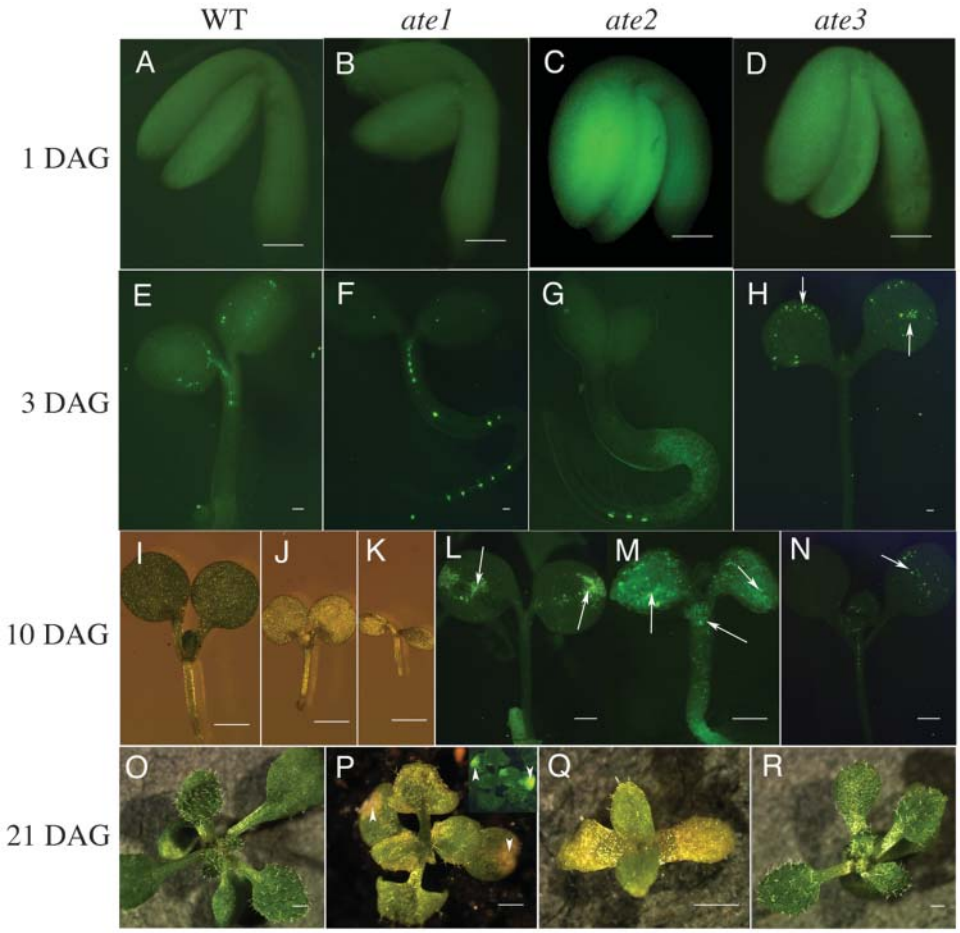

Figure 2. Expression pattern of the *Atxyn3*::*YFP* marker gene and phenotypes of seedlings. Wild type (A, E, I, and O), *ate1* (B, F, J, L, and P), *ate2* (C, G, K, M, and Q), and *ate3* (D, H, N, and R) are shown. Seedlings were grown for 1 d (A–D), 3 d (E–H), 10 d (I–N), 14 d (Q), and 21 d (O, P, and R). Arrows in H, L, M, and N show ectopic *Atxyn3*::*YFP* marker expression. Arrowheads indicate the ectopic *Atxyn3*::*YFP* marker gene expression (P, inset), and dying cell region (P). Scale bars, 100 μm (A–H) and 1 mm (I–R).

I–K and O–Q). The plant size of the *ate1* mutants was about 70% of the wild type. The *ate2* mutant did not germinate in the soil, but it could germinate and produce up to four to five rosette leaves on the agar plates (Fig. 2Q). About 2 weeks after germination, *ate2* plants had a diameter of about 1 cm and died without producing any floral buds. The morphology of *ate3* plants was almost the same as that of wild-type plants (Fig. 2, O and R). Cell death was also observed in the *YFP*-expressing cells at about 3 weeks after germination of the *ate1* and *ate3* mutants, and at about 1 to 2 weeks after germination of the *ate2* mutant (Fig. 2P).

Lignin Accumulation in the *ate* Mutants

Lignin, a complex phenylpropanoid polymer, is a characteristic component of TEs, and it can be visualized by using UV illumination or the lignin-staining dye, phloroglucinol-HCl (Fig. 3, A and B). To examine whether *ate* mutants produce ectopic TEs, we examined the pattern of lignin deposition in their leaves and found ectopic lignin deposition occurred in some parenchyma cells (Fig. 3, C, D, and H–M). In some cases, a striped pattern of secondary walls was observed in these cells (Fig. 3, C, D, and G). The ectopic lignin-accumulating region was consistent with the ectopic *YFP*-expressing region (Fig. 3, E–G). In the *ate1* mutant, patches of cells with ectopic lignin accumulation were also observed (Fig. 3, I and L). These results suggest that the parenchyma cells of these mutants transdifferentiated into TEs.

Effect of the *ate* Mutation on the Induction of TE Transdifferentiation

Our finding that the *ate* mutations cause ectopic induction of TEs prompted us to investigate further the potential of parenchyma cells to transdifferentiate into TE cells in *ate* mutants. We developed an in vitro system, in which parenchyma cells transdifferentiate into TE cells by incubating the hypocotyl on liquid medium containing brassinosteroids (H. Fukuda, unpublished data). In the wild type, ectopic TEs were transdifferentiated at 5 to 6 d after treatment, when they were identified by the presence of helical secondary wall thickenings. In the *ate* mutants, TE transdifferentiation was observed 3 to 4 d after treatment (Fig. 4, A and B), and the transdifferentiation was more efficient as compared with the wild type (Fig. 4C). This result suggests that the *ate* mutations increase the sensitivity of the cells to the induction of TE transdifferentiation, which might lead to the formation of the ectopic TEs.

Ectopic Expression of Another TE Marker Gene in the *ate1* Mutant

To examine the effect of the *ate1* mutation on TE formation steps in planta, we assessed the expression pattern of another xylem-specific marker, the

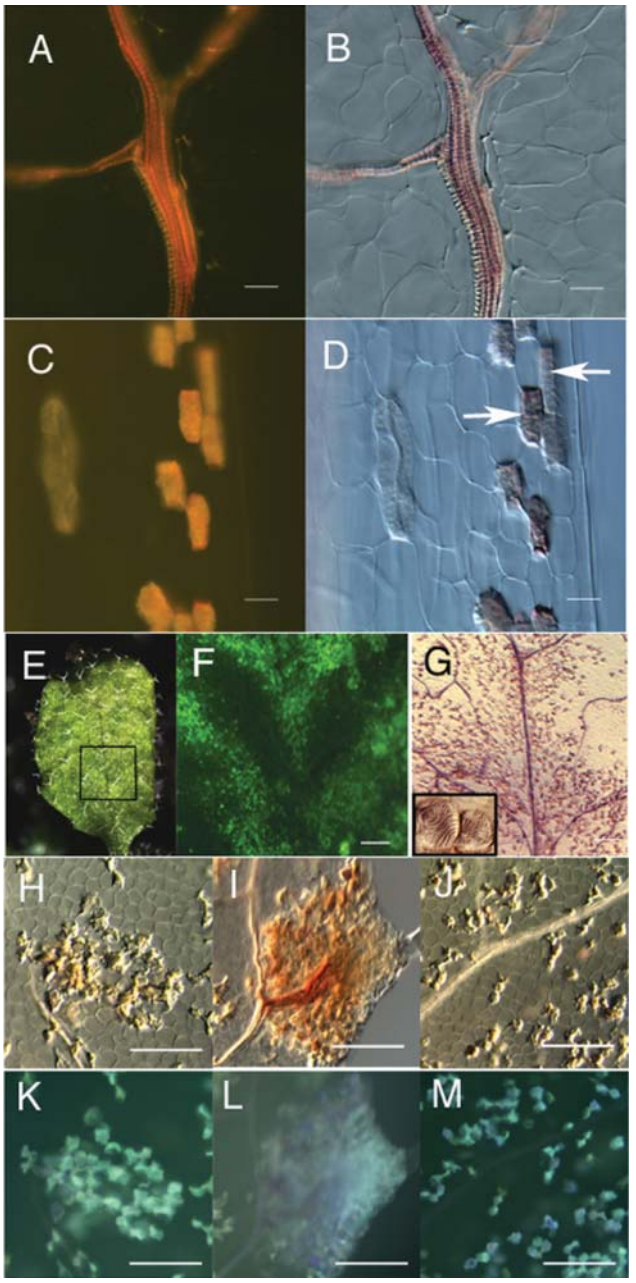

Figure 3. Observation of the lignin deposition in rosette leaves of 3-week-old plants. Wild type (A and B), *ate3* (C–G), *ate1* (H, I, K, and L), and *ate2* (J and M) are shown. Lignin was visualized by phloroglucinol-HCl staining (B, D, G, and H–J) or UV illumination (A, C, and K–M). A region of the daylight image of *ate3* leaf (E, inset) is magnified in F (YFP fluorescent) and G (phloroglucinol staining). The inset of G shows ectopically formed lignified cells with spiral secondary wall thickenings, which indicates TE formation. White arrows in D also indicate ectopically formed TEs with spiral lignin deposition. Scale bars, 25 μm (A–D) and 100 μm (F–M).

pAt3g62160::*YFP* chimeric gene in the *ate1* mutants. *At3g62160* encodes a putative acetyltransferase and exhibits significant sequence similarity to *Z3714* and *Z9029*, which are up-regulated in the TE maturation steps in the zinnia TE transdifferentiation

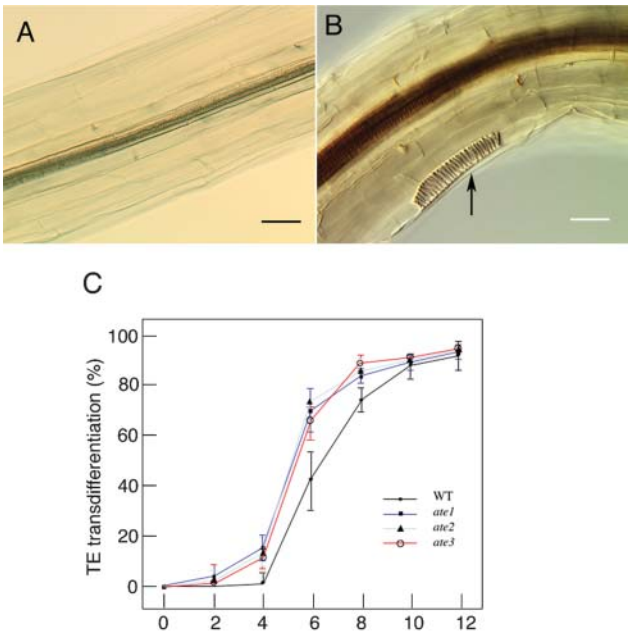

Figure 4. In vitro TE induction in the *ate* mutants. Hypocotyls of the wild type (A) and the *ate1* mutant (B) at 4 d after treatment are shown. The frequency of TE transdifferentiation in the *ate* mutants is shown (C). Twenty-five hypocotyls were examined in each sampling point, and the SD was calculated from data of six independent experiments. Arrows indicate ectopic transdifferentiated TE cell. Scale bars, 50 μm (A–F).

system (Demura et al., 2002). *YFP* expression in the *pAt3g62160*::*YFP* transgenic plant was observed only in developing TEs (Fig. 5, A and C). The *ate1* mutation was introduced into the *pAt3g62160*::*YFP* transgenic plant by crossing, and *YFP* was also ectopically expressed, as seen in the *ate1 Atxyn3*::*YFP* plants (Fig. 5, B and D).

Effect of the *ate* Mutations on the Expression of TE Formation-Related Genes

Next, we examined the expression levels of TE formation-related genes in the *ate* mutants. A semiquantitative reverse transcription (RT)-PCR experiment was performed (Sawa et al., 2002) because there is sequence similarity between the examined genes and their family genes in Arabidopsis and because probes specific to the untranslated region of these genes did not give a clear signal in the northern analysis. The PCR band corresponding to the endogenous *Atxyn3* gene demonstrated an obvious, increased intensity in the *ate* mutants compared with that of the wild type (Fig. 6), indicating that the *ate* mutations enhance not only the reporter *YFP* expression but also the endogenous *Atxyn3* gene expression. The expression of *XCP1*, a Cys protease gene that is known to be expressed specifically in the cell death steps of TEs, was also up-regulated in all of the *ate* mutants (Fig. 6). Expression of the laccase (*At2g38080*) and cellulose synthase genes (*IRX3*, *At5g17420*), which are also related to the secondary wall formation of TEs, were up-regulated in the *ate* mutants. Furthermore, we examined the expression level of the *At1g03820* gene in *ate* mutants because *At1g03820* encodes a putative arabinogalactan-protein and exhibits a significant sequence similarity to the *TED3* gene, which is a marker gene of TE precursor cells (Demura and Fukuda, 1994). The intensity of the PCR band corresponding to the *At1g03820* gene clearly increased in the *ate* mutants compared with the wild type (Fig. 6).

Effect of the *ate1* Mutation in Vascular Pattern Formation

We investigated the role of the *ATE1* gene in the determination of vascular patterning. It is well known that auxin affects vascular patterning and xylem development (Sachs, 1991, 2000; Tuominen et al., 1997). To examine the effects of the *ate1* mutation on auxin distribution, we introduced the *DR5*::*GUS* (β-glucuronidase) reporter gene into the *ate1* mutant. The *DR5*::*GUS* gene expression pattern in the *ate1* mutant was almost the same as that of wild type, and the *ate1* mutation did not affect the vascular patterning (Fig. 7, A and B). Furthermore, when the *pin1* mutation was introduced into the *ate1* mutant, the *pin1* venation pattern was not affected (Fig. 7, C and D). We also assessed the effects of the *ate1* mutation in the discontinuous vascular network phenotype of the *van3* mutants and found that the *van3* phenotype was not

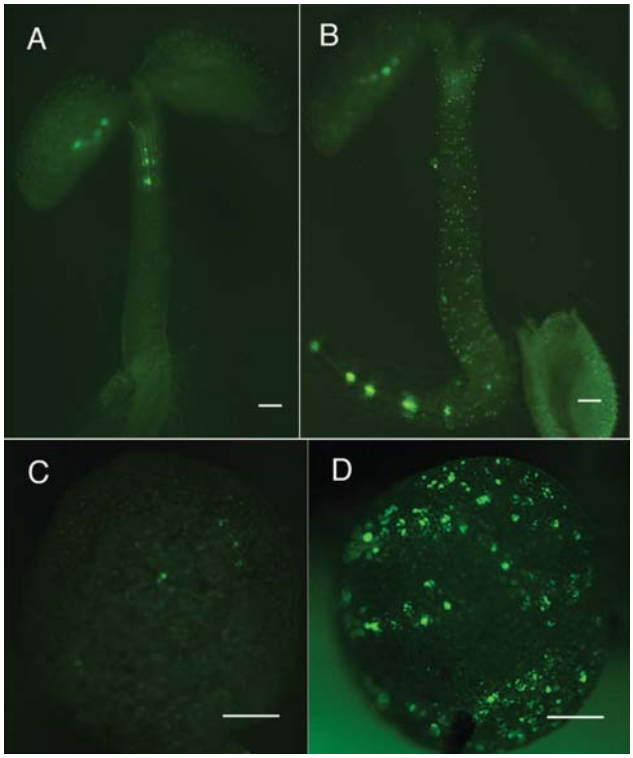

Figure 5. *pAt3g62160*::*YFP* marker gene expression. Wild type (A and C) and the *ate1* mutant (B and D) are shown. Note the ectopic distribution of fluorescence signals in the hypocotyl (B) and cotyledon (D) of the *ate1* mutant. Scale bars, 500 μm (A–D).

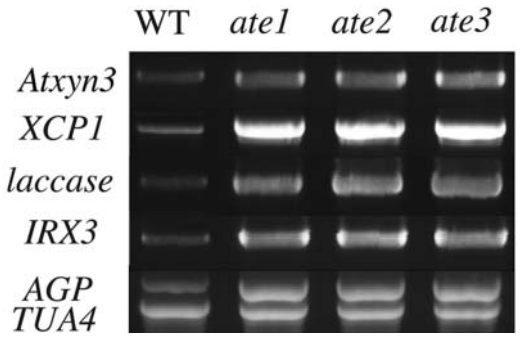

Figure 6. Expression analysis of TE formation-related genes in the *ate* mutants. Two pairs of primers in the same reaction mixture were used in the quantitative RT-PCR experiment. One pair of primers was used to amplify the internal control gene, *TUA4*, while other gene-specific primers were used to amplify *Atxyn3*, *XCP1*, laccase (*At2g38080*), *IRX3*, or *AGP* (*At1g03820*). Only the *TUA4* control for the *AGP* amplification is shown.

affected by the *ate1* mutation (Fig. 7, E and F). These results suggest that the *ATE1* gene is not involved in venation pattern construction.

DISCUSSION

In spite of its importance, very little is known about the molecular mechanisms of transdifferentiation and the masking system in planta. Here, we isolated and characterized mutants that may be involved in the masking of transdifferentiation in situ.

Ectopic Lignification in the *ate* Mutants

We isolated three novel *ate* mutants of Arabidopsis showing an ectopic expression of a TE marker gene. Ectopic lignification was also observed in the cells expressing the TE marker gene in the *ate* mutants. Ectopic lignification is a characteristic phenotype of *ectopic lignification1* and *lion tale*, which are defective in cell expansion (Cano-Delgado et al., 2000), *radially swollen 1*, which is defective in a catalytic subunit of cellulase synthase (Arioli et al., 1998), and *korrigan*, which is defective in a plasma membrane-associated β-1,4-endoglucanase (Nicol et al., 1998). The varying degree of lignification observed in these mutants was correlated with the degree of altered cell expansion, suggesting a meaningful link between the processes of cell expansion and lignification (Cano-Delgado et al., 2000). This linkage is clearly demonstrated by the temperature dependence of both cell expansion and ectopic lignification in the *radially swollen 1* allele (Arioli et al., 1998). Ectopic lignification in these mutants could be the secondary effect due to the modulation of cell wall organization. As for the *ate* mutants, we did not detect alterations in cell shape and cell division, even in the cells ectopically expressing the TE marker gene. Furthermore, the expression profile of genes involved in cellulose synthesis and defense responses, not the TE formation-related genes, are obviously affected in the *ectopic lignification* mutant (Cano-Delgado et al., 2003), although the *ate* mutants have a significant increase in transcription of TE formation-related genes. This suggests that the *ate* mutants with an ectopic lignification are included in the novel category mutants.

ATE Genes in Vascular Pattern Formation

Recently, several factors have been investigated for their possible roles in vein patterning. Auxin is a pivotal molecule that controls the vein patterning. The auxin canalization hypothesis presented by Sachs (1991) has been supported by recent genetic analyses. In addition, the involvement of factors other than auxin in vein patterning has been suggested, as typically shown in *van3* mutants (Koizumi et al., 2000). However, all of the *ate* mutants showed the normal

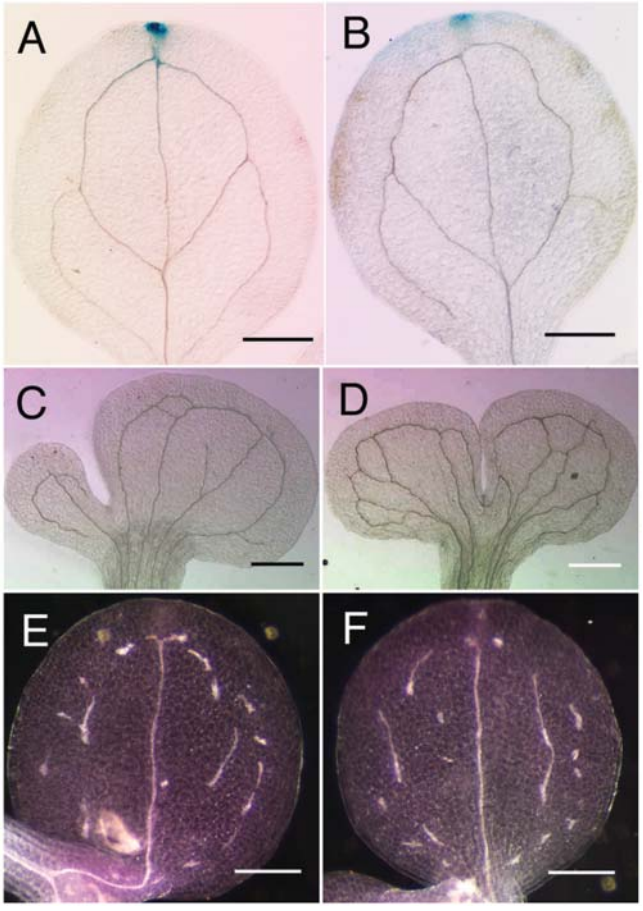

Figure 7. Genetic analyses of the *ate1* mutant. Histochemical localization of GUS activity in the cotyledon of the 10-d-old wild type (A) and *ate1* mutant (B) carrying the *pDR5*::*GUS* gene is shown. Cotyledon vein patterns of the 10-d-old *pin1* (C), *pin1 ate1* (D), *van3* (E), and *van3 ate1* (F) mutants are shown. The *ate1* mutation was confirmed by ectopic YFP signals (data not shown). Light field (A–D) and dark field microscopy was used to clarify the discontinuous vein pattern (E and F). Scale bars, 500 μm (A–F).

venation pattern, and the *ate1* mutation did not affect the expression pattern of the *DR5*::*GUS* marker gene, the *pin1* mutant phenotype, or the *van3* mutant phenotype. These results indicate that the *ATE* genes do not affect the determination of the venation pattern.

ATE Genes in the TE Transdifferentiation System

All of the *ate* mutants showed the ectopic marker gene expression in all organs tested, i.e. the roots, hypocotyls, cotyledons, and leaves for *ate1* to *3*, and sepals, petals, stamens, and carpels for *ate1* and *ate3*. Spatial association of the ectopic YFP signal region, with ectopic lignin deposition and cell death region, was also observed in the *ate* mutants. Furthermore, we did not observe the ectopic callus formation, altered cell shape, and abnormal cell division in the *ate* mutants. Together with the result of semiquantitative RT-PCR, it was demonstrated that some parenchyma cells in the *ate* mutants transdifferentiate into TEs in situ. A master key gene, which determines the identity of each cell type, is expected to be activated in a transdifferentiation system. However, we showed here that the recessive *ate* mutations ectopically induce transdifferentiation into TEs. This result implies that there is a repression system for transdifferentiation into a specific cell type, and therefore, the masking of transdifferentiation is an active mechanism in planta, although we do not deny the possibilities that the *ATE* genes are responsible for the regulation of BR sensitivity, biosynthesis, or metabolisms because *ate* mutants showed rapid TE transdifferentiation in the in vitro TE transdifferentiation system.

Morphologically, we could not detect ectopic formation of cell types other than TEs in *ate* mutants. However, there is a possibility of a transdifferentiation into other types of cells such as xylem parenchyma cells. These are observed in the zinnia xylogenic culture system as a minor population of transdifferentiated cells, as opposed to a major population of TEs (Shinohara et al., 2000). We may need to examine transdifferentiated cells using a cell type-specific molecular marker in the *ate* mutants. Nevertheless, distinct morphological features are essential for the function of each type of vascular cell. Therefore, the absence of newly formed cells with distinct morphology (apart from TEs) in the *ate* mutants strongly suggests that transdifferentiation occurs only into TEs. Interestingly, all three recessive *ate* mutants showed transdifferentiation only into TEs. This finding implies the presence of a positive TE-specific masking system in situ. If the masking system of transdifferentiation in situ is the common system in all cell types, and if the *ATE* genes regulate the common system, we would expect to observe ectopic transdifferentiation of TEs and various other types of cells in the *ate* mutants. This may not be so surprising because TEs are induced preferentially in in vitro cultures (Fukuda, 1992). However, only the ectopic TEs are transdifferentiated in the *ate* mutants, and *ATE* genes would be responsible only for the masking of transdifferentiation into the TEs. Fukuda (1997) has suggested that wounding and a combination of auxin and cytokinin are a prerequisite for TE transdifferentiation in vitro. However, TE transdifferentiation occurs in the *ate* mutants without exogenous phytohormones or wound stress, which suggests that the *ATE* genes may be crucial for the initiation of TE transdifferentiation downstream of the auxin/cytokinin signaling. Identification and characterization of the *ATE* genes will provide further insights into the molecular mechanism of transdifferentiation masking in situ.

CONCLUSION

Here we characterized the *ate* mutants. From the analysis of the *ate* mutants, we suggested the presence of a transdifferentiation-repressing system, and we provided a new model that plants may prepare cell type-specific transdifferentiation-masking system. Our approach using a TE-specific gene marker led to the isolation of a new type of mutant in which TE transdifferentiation is induced ectopically. This approach may be applicable for analyzing the transdifferentiation-masking system of other types of cells, tissues, and/or organs by using their specific marker genes. The identification and characterization of genes involved in the transdifferentiation machinery will elucidate the general mechanism of masking of transdifferentiation in planta.

MATERIALS AND METHODS

Isolation of Mutants

The *YFP* cDNA fragment was ligated with the *Atxylanase 3* promoter region, which includes a 2,000-bp region upstream of the predicted start codon of *Atxylanase 3*. The resulting *Atxyn3*::*YFP* gene was transformed into Agrobacterium strain MP90 and further introduced into wild-type Columbia. Seeds of *Atxyn3*::*YFP* transgenic plants were mutagenized in 0.3% ethane methyl sulfonate (Sigma, St. Louis) for 20 h. The self-fertilized progeny of these seeds were collected, and 10,000 plants were screened for ectopic marker gene expression after 14 d growth on germination medium (0.5× Murashige and Skoog basal salts [Wako], pH 5.7). Seedlings were examined under a microscope using fluorescent light illumination (FLIII; Leica, Wetzlar, Germany).

Histological Analysis

Samples were fixed overnight in a 9:1 mixture of ethanol and acetic acid at room temperature for whole-mount observation. Fixed samples were cleared in a mixture of chloral hydrate, glycerol, and water solution (8 g:1 mL:2 mL) and observed under a light microscope equipped with Nomarski optics (U-DICT; Olympus, Tokyo).

Histochemical Localization of GUS Activity

The *ate1* mutation was introduced into the *pDR5*::*GUS* transgenic plants by crossing. Samples were fixed in 90% (v/v) acetone for at least 60 min on ice. After washing in water 3 times, they were immersed in a reaction mixture containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronide, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide in 100 mM sodium phosphate buffer, pH 7.2, and incubated for 4 h (*pAthb8*::*GUS*) or 24 h (*DR5*::*GUS*)

in the dark. After the reaction, samples were mounted with a mixture of chloral hydrate, glycerol, and water and observed under a light microscope equipped with Nomarski optics (BX-50; Olympus).

For the lignin observation, samples were mounted with a mixture of chloral hydrate, glycerol, water, and 1% Phloroglucinol (Sigma) in hydrochloric acid (8 g:1 mL:2 mL:50 μL) and observed under a light microscope equipped with Nomarski optics or examined under a microscope using UV illumination (BX-50; Olympus).

Genetic Mapping

Genomic DNA was extracted from 500 (*ate1*), 96 (*ate2*), and 24 (*ate3*) individual F2 mutant seedlings generated from crosses of each mutants to ecotype Landsberg *erecta*. The DNA samples were genotyped using several microsatellite loci that are polymorphic between ecotypes Landsberg *erecta* and Columbia (Bell and Ecker, 1994), allowing us to map the chromosomal position of each *ate* mutant locus.

In Vitro TE-Inducible Experiment

Arabidopsis (*Arabidopsis thaliana*) plants were grown on agar plates for 5 d in the dark. The hypocotyls were floated on a medium containing 4.41 g Murashige and Skoog medium (Wako), 10 μg thiamin HCl, 5 μg nicotinic acid, 10 μg pyridoxine HCl, 100 μg myo-inositol, 2 μg Gly, 0.5 g MES, 0.5 mg 2, 4-dichlorophenoxyacetic acid, 50 μg kinetin, and 1 μmol brassinolide per liter. After incubation for various periods (2, 4, 6, 8, and 10 d), 25 samples were fixed in each sampling point. Fixation was performed overnight in a 9:1 mixture of ethanol and acetic acid at room temperature for whole-mount observation. Fixed samples were cleared in a mixture of chloral hydrate, glycerol, and water solution (8 g:1 mL:2 mL) and observed under a light microscope equipped with Nomarski optics (U-DICT; Olympus), and the number of hypocotyls containing cells showing spiral lignin deposition were counted.

RT-PCR Analysis

Two pairs of primers in the same reaction mixture were used in the quantitative RT-PCR experiment. RT-PCR analysis for the quantification of the endogenous *Atxyn3* transcript was performed according to the instructions for the First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ) using a set of endogenous gene specific primers (5′-TGTTTGTTCTGCTCTTGATATGCTC-3′, 5′-CAAAGAAGAGAGATCAATGGAGATA-3′) and a set of primers specific to the internal control gene, *TUA4* (5′-CTTCCTTGACTGCTTCTC-3′, 5′-TCATCGTCACCACCTTCA-3′), in the same reaction mixture. We used primer sets to amplify the transcripts of *XCP1*, 5′-GAGGCTTCAGGAAGAGACTTCCAG-3′, 5′-CACTTGGTCTTGGTAGGATATGAGG-3′; laccase (*At2g38080*), 5′-GGTGGATGGGTCGTCATGAGATTC-3′, 5′-CGTGGCGTGATGTTGATATGTCGCCC-3′; *IRX3*, 5′-GGCGTTGTTGCAGGCATCTCAG-3′, 5′-CAGCAGTTGATGCCACACTTGG-3′; and *AGP* (*At1g03820*), 5′-GACAATGGGAGAGGTTACGGTAATG-3′, 5′-CTAAGGCTCATACTCTTCTTG-3′.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AB194395.

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