The N-end rule pathway controls multiple functions during *Arabidopsis* shoot and leaf development

Emmanuelle Graciet[a,b,1], Franziska Walter[a], Diarmuid Ó Maoiléidigh[a], Stephan Pollmann[c], Elliot M. Meyerowitz[b,1], Alexander Varshavsky[b,1], and Frank Wellmer[a,b,1]

[a]Smurfit Institute of Genetics, Trinity College Dublin, Dublin 2, Ireland; [b]Division of Biology, California Institute of Technology, Pasadena, CA 91125; and [c]Department of Plant Physiology, Ruhr-University Bochum, 44801 Bochum, Germany

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The ubiquitin-dependent N-end rule pathway relates the in vivo half-life of a protein to the identity of its N-terminal residue. This proteolytic system is present in all organisms examined and has been shown to have a multitude of functions in animals and fungi. In plants, however, the functional understanding of the N-end rule pathway is only beginning. The N-end rule has a hierarchic structure. Destabilizing activity of N-terminal Asp, Glu, and (oxidized) Cys requires their conjugation to Arg by an arginyl–tRNA–protein transferase (R-transferase). The resulting N-terminal Arg is recognized by the pathway's E3 ubiquitin ligases, called "N-recognins." Here, we show that the *Arabidopsis* R-transferases AtATE1 and AtATE2 regulate various aspects of leaf and shoot development. We also show that the previously identified N-recognin PROTEOLYSIS6 (PRT6) mediates these R-transferase-dependent activities. We further demonstrate that the arginylation branch of the N-end rule pathway plays a role in repressing the meristem-promoting *BREVIPEDICELLUS* (*BP*) gene in developing leaves. *BP* expression is known to be excluded from *Arabidopsis* leaves by the activities of the ASYMMETRIC LEAVES1 (AS1) transcription factor complex and the phytohormone auxin. Our results suggest that AtATE1 and AtATE2 act redundantly with AS1, but independently of auxin, in the control of leaf development.

arginine transferase | plant | protein degradation

In eukaryotes, the control of protein stability is carried out largely by the ubiquitin (Ub) system, which mediates the conjugation of the 8-kDa protein Ub to target proteins, marking them for proteolysis. The selectivity of ubiquitylation is mediated primarily by E3 Ub ligases, which recognize specific degradation signals (degrons) of substrate proteins (1). Regulated proteolysis by the Ub system underlies just about every cellular and organismal function in eukaryotes. In plants, Ub-dependent processes play major and diverse roles, including the regulation of signaling by phytohormones, such as auxin, gibberellins, and jasmonic acid (2).

An essential determinant of one class of degrons, called "N-degrons," is a substrate's destabilizing N-terminal residue. The set of destabilizing residues yields a rule, called the "N-end rule," which relates the in vivo half-life of a protein to the identity of its N-terminal residue (3–6). The N-end rule has a hierarchic structure (Fig. 1). In eukaryotes, N-terminal Asn and Gln are tertiary destabilizing residues in that they function through enzymatic deamidation to yield the secondary destabilizing residues Asp and Glu. The activity of Asp and Glu, and also of (oxidized) Cys, requires their conjugation by arginyl–tRNA–protein transferase (R-transferase) to Arg, one of the primary destabilizing residues (Fig. 1) (4, 5, 7). Whereas in both the yeast *Saccharomyces cerevisiae* and the mouse, an R-transferase is encoded by a single gene (7, 8), the model plant *Arabidopsis thaliana* contains 2 closely related R-transferases: AtATE1 (At5g05700) and AtATE2 (At3g11240) (9). Primary destabilizing residues are recognized by E3 Ub ligases of the N-end rule pathway, called "N-recognins" (10–12). Although a single N-recognin is present in *S. cerevisiae* (13), mammalian genomes encode at least 4 distinct N-recognins (11, 12). In plants, 2 N-recognins, termed PROTEOLYSIS 1 (PRT1) and PRT6, have been identified in *Arabidopsis* (10, 14–16), but other N-recognins are likely to be present as well (10, 16, 17).

Whereas in animals and fungi the N-end rule pathway is known to mediate the control of diverse cellular and developmental processes (4, 18–20), its functions in plants are only beginning to emerge. Yoshida et al. (9) have demonstrated that the R-transferase-coding gene *AtATE1* is disrupted in the *Arabidopsis* mutant *delayed leaf senescence1* (*dls1*), in which leaf senescence is abnormally slow. Recently, it was shown that *AtATE1* and *AtATE2* are also involved in promoting seed germination and establishment through the removal of sensitivity to the hormone abscisic acid (21). This process requires the N-recognin PRT6 (21), which recognizes N-end rule substrates with basic N-terminal residues, including Arg (10).

In the present study, we characterized a double mutant lacking both of the *Arabidopsis* R-transferases. We describe several lines of evidence that reveal an involvement of the arginylation branch of the N-end rule pathway in the control of shoot and leaf development.

Results

***ate1 ate2* Mutant Plants Exhibit Abnormal Shoot and Leaf Development.** To assess the function of R-transferases in plant development, we isolated T-DNA insertion lines (*ate1-2* and *ate2-1*, referred to hereafter as *ate1* and *ate2*, respectively) for the genes encoding the *Arabidopsis* R-transferases AtATE1 and AtATE2. To test whether R-transferase activities are altered in these lines, we used a previously validated in vitro arginylation assay (7). Both *ate1* and *ate2* single mutants retained a fraction of the wild-type arginylation activity (Fig. 2*A*), indicating that both genes contribute to the pool of active R-transferase. In contrast, no R-transferase activity was detected in extracts from *ate1 ate2* double-mutant seedlings, strongly suggesting that *AtATE1* and *AtATE2* encode the entire repertoire of R-transferases in *Arabidopsis* and that the corresponding T-DNA insertions in *AtATE1* and *AtATE2* resulted in functionally null mutants. Another conclusion from the above tests was that AtATE1 accounts for a much higher fraction of the overall R-transferase activity than AtATE2.

We compared *ate1* and *ate2* single mutants as well as *ate1 ate2* double mutants with wild-type plants at different stages of development and in different growth conditions. In contrast to *ate1* and *ate2* single mutants, which resembled wild-type plants, *ate1 ate2* double mutants exhibited a variety of abnormal phe-

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[1]To whom correspondence may be addressed. E-mail: graciete@tcd.ie, meyerow@caltech.edu, avarsh@caltech.edu, or wellmerf@tcd.ie.

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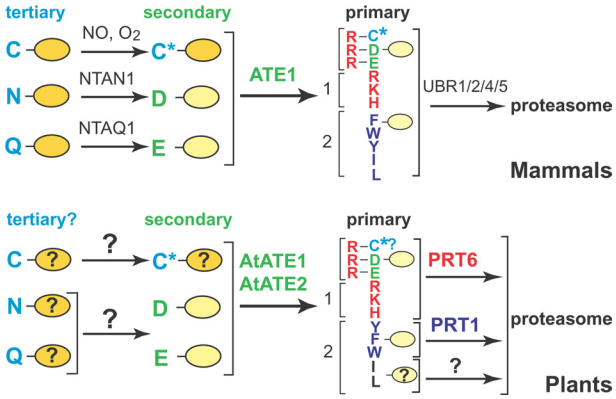

Fig. 1. The N-end rule pathway in mammals and plants. N-terminal amino acid residues are indicated by single-letter abbreviations. Yellow ovals denote the rest of a protein substrate. Primary, secondary, and tertiary denote distinct subsets of destabilizing N-terminal residues. C* represents oxidized Cys. Primary destabilizing residues are recognized in mammals by N-recognins of the UBR family (11, 12). In plants, aromatic hydrophobic type-2 residues are recognized by PRT1 (16), whereas basic type-1 residues are recognized by PRT6 (10).

notypes, which were observed in both short-day conditions and continuous light, but were considerably stronger under short-day conditions. Leaves of wild-type plants were relatively flat and had only slightly serrated margins (Fig. 2*B*), whereas rosette leaves of *ate1 ate2* plants were wavy, had deeper serrations, and were slightly lobed (Fig. 2*C*). The severity of the leaf defects of *ate1 ate2* mutants gradually increased, so that leaves formed during late stages of vegetative development were much more affected than leaves arising early. Under short-day conditions (and, to a lesser extent, under continuous light), axillary meristems of *ate1 ate2* double-mutant plants produced leaves before the transition to flowering (Fig. 2*E*), indicating a loss of apical dominance compared with the wild type (Fig. 2*D*).

In wild-type plants, the initiation of lateral branches and flowers follow a defined radial pattern (phyllotaxis), with increases in the internode distances. In contrast to wild-type plants, *ate1 ate2* double mutants exhibited defects in both phyllotaxis (33.3% of *ate1 ate2* plants; $n = 36$) and internode elongation (41.7% of *ate1 ate2* plants; $n = 36$) (Fig. 2*F*). The phyllotaxis defects could stem from either abnormal initiation or growth defects that occurred after initiation (e.g., through twisting), whereas the decrease of internode elongation could be caused by abnormal cell elongation and/or cell division. Scanning electron microscopy of mutant stems revealed patches of small cells (Fig. 2*H*) that were not present in the wild type (Fig. 2*G*), implying that cell elongation is affected in the double mutant. Additionally, stems of *ate1 ate2* plants grown in short-day conditions and then transferred to continuous light to synchronously induce flowering (Fig. S1*A*) were significantly shorter than those of the wild type (Fig. 2 *I* and *J* and Fig. S1*B*). Treatment of the double mutant with the gibberellin GA_3, which is known to promote cell elongation (22), resulted in a partial rescue of this defect (Fig. S1*B*), again suggesting abnormal cell elongation in *ate1 ate2* plants. Finally, *ate1 ate2* double mutants displayed delayed leaf senescence in the dark and reduced seed germination rates, as described previously (9, 21).

To determine whether the abnormal phenotypes were indeed caused by the absence of R-transferase activity, we transformed *ate1 ate2* plants with fragments of genomic DNA containing either *AtATE1* or *AtATE2*. Transformants (18 and 6 independent lines for *AtATE1* and *AtATE2*, respectively) characterized in short-day conditions were morphologically similar to wild-type plants (Fig. S2). We therefore conclude that the defects observed in *ate1 ate2* plants are caused by the absence of R-transferase activity.

In summary, the analysis of *ate1 ate2* double-mutant plants and of the corresponding single mutants showed that *AtATE1*

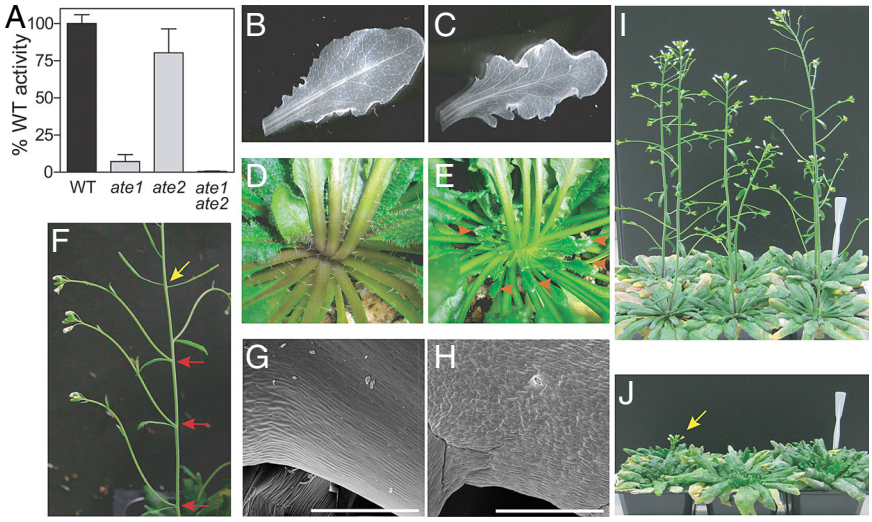

Fig. 2. *AtATE1* and *AtATE2* act redundantly in the control of plant development. (*A*) Loss of R-transferase activity in *ate1 ate2* mutant seedlings. R-transferase activities in different mutant backgrounds were examined in vitro. The assay measures the conjugation of [^{3}H]Arg to bovine α-lactalbumin, which bears N-terminal Glu, a substrate of R-transferases. Wild-type R-transferase activity was set to 100%. Activities are represented as a percentage of wild-type activity. Error bars represent standard errors calculated based on 6 independent measurements obtained with 2 different protein extracts. (*B* and *C*) Cleared wild-type (*B*) and *ate1 ate2* double-mutant (*C*) leaves from plants grown in short-day conditions. Note the lobes and wavy leaf margins in *C*. (*D* and *E*) Wild-type (*D*) and *ate1 ate2* double-mutant (*E*) plants grown for 3 months in short-day conditions. The *ate1 ate2* mutants show early outgrowth of axillary meristems, as indicated by the formation of leaves in the axils of rosette leaves (arrowheads). (*F*) Phyllotaxis (red arrows) and internode elongation defects (yellow arrow) in *ate1 ate2* double mutants. (*G* and *H*) Scanning electron micrograph of part of a stem from a wild-type (*G*) and an *ate1 ate2* plant (*H*), respectively. Note the presence of patches of small cells in the double mutant. (Scale bars: 500 μm.) (*I* and *J*) Wild-type and *ate1 ate2* mutant plants were grown in short-day conditions for 2 months and transferred to continuous light for a synchronous induction of flowering (see Fig. S1*A*). After transfer to inducing conditions, stems of *ate1 ate2* double-mutant plants (*J*) exhibited reduced elongation compared with those of the wild type (*I*). The arrow in *J* points to an inflorescence with mature flowers. Pictures were taken 19 days after transfer to continuous light.

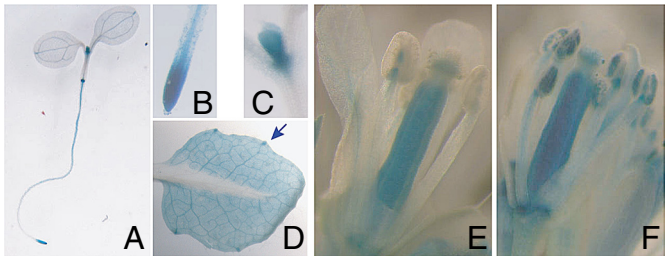

Fig. 3. Expression patterns of *AtATE1* and *AtATE2*. *AtATE1* and *AtATE2* GUS translational fusions were introduced into wild-type plants. T2 and T3 plants were stained at different stages of development to detect GUS expression. (*A–D*) *AtATE1* reporter activity was detected in 5-day-old seedlings (*A*), especially in root (*B*) and shoot (*C*) apices, as well as in the vasculature and in hydathodes (arrow) of more mature leaves (*D*). (*E* and *F*) In flowers, *AtATE1* reporter activity was found mainly in carpels and the connective tissue of anthers (*E*), whereas *AtATE2* reporter activity was also detected in pollen grains (*F*).

and *AtATE2* act in a redundant manner and control various processes during leaf and shoot development.

***AtATE1* and *AtATE2* Have Similar Expression Patterns.** To survey the expression patterns of *AtATE1* and *AtATE2*, we used genomic fragments that allowed complementation of the *ate1 ate2* double mutant to construct translational β-glucuronidase (GUS) reporters. A total of 6 of 7 and 2 of 4 independent transformants obtained for *AtATE1* and *AtATE2* reporter constructs, respectively, showed strong GUS-specific staining. A detailed analysis of the reporter lines indicated that *AtATE1* and *AtATE2* have similar expression patterns (Fig. 3 and Fig. S3), which is consistent with the functional redundancy of the two R-transferases. In seedlings, we detected GUS activity for both reporter lines in the root apex, in emerging lateral root primordia, as well as in the shoot apex and in young leaves (Fig. 3 *A–C* and Fig. S3). In older plants, we observed GUS activity in expanding leaves, with a stronger staining in veins and hydathodes (Fig. 3*D*). After the transition to flowering, we detected GUS-specific staining in the inflorescence stem, in the axils of lateral branches and flowers (Fig. S3), and in young floral buds and mature flowers (Fig. 3 *E* and *F*). Although *AtATE1* and *AtATE2* have similar expression patterns throughout most of plant development, differences were found in mature flowers, in which *AtATE2* was expressed in mature pollen grains (Fig. 3*F*), in contrast to *AtATE1* (Fig. 3*E*).

In summary, *AtATE1* and *AtATE2* are most strongly expressed in tissues that are characterized by rapid growth, in good agreement with the phenotypic alterations in *ate1 ate2* double-mutant plants.

***ate1 ate2* Mutant Phenotypes Are Due to a Disruption of the N-End Rule Pathway.** It is possible that the functions of R-transferases are not confined to the N-end rule pathway, in that N-terminal arginylation of some proteins may alter their functional activity without a change in their in vivo half-life (23). To test this possibility, we analyzed 3 mutant alleles of the previously identified N-recognin PRT6, which recognizes N-end rule substrates with N-terminal basic residues and should function downstream of R-transferases (Fig. 1) (10, 21). If some or all of the *ate1 ate2* phenotypes were indeed caused by a disruption of the arginylation branch of the N-end rule pathway, then the *prt6* mutant alleles should show similar phenotypic alterations. In agreement with this idea, we found that *prt6* mutants resembled *ate1 ate2* double mutants in that they showed comparable defects in the development of shoots and leaves (Fig. 4 and Fig. S4). These phenotypes were weaker than those of the *ate1 ate2* double

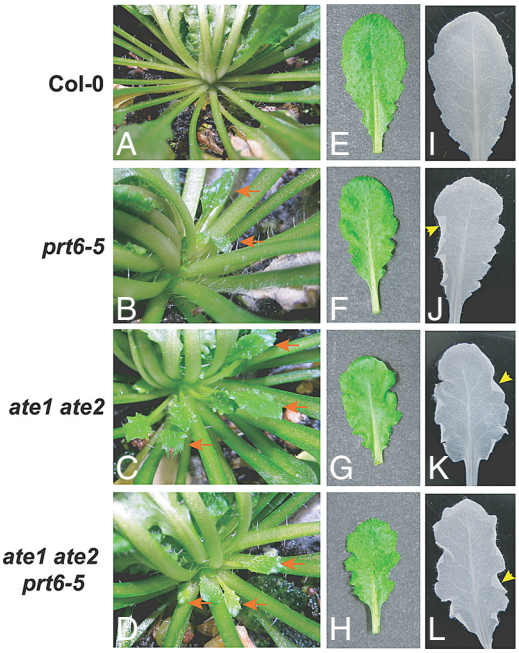

Fig. 4. Phenotypes of *ate1 ate2* plants result from a disruption of the N-end rule pathway. Pictures of 70-day-old plants grown in short-day conditions. (*A–D*) In *prt6-5* (*B*), *ate1 ate2* (*C*), and *ate1 ate2 prt6-5* plants (*D*), but not in the wild type (*A*), leaves formed in the axils of rosette leaves (arrows), indicating loss of apical dominance. (*E–L*) Contrary to leaves from the wild type (*E* and *I*), the leaf margins of *prt6-5* (*F* and *J*), *ate1 ate2* (*G* and *K*), and *ate1 ate2 prt6-5* plants (*H* and *L*) were lobed and wavy (arrowheads). The leaves shown in *I–L* were cleared.

mutant, although 2 of the alleles tested (*prt6-1* and *prt6-5*) are likely null mutants (the predicted gene product in these lines lacks the functionally essential RING domain). Because the set of known *Arabidopsis* N-recognins is probably incomplete (10), it is possible that another (as yet unidentified) N-recognin could partially compensate for the loss of PRT6 function. To further address functional links between PRT6 and R-transferases, we constructed an *ate1 ate2 prt6-5* triple mutant. The resulting plants (Fig. 4 *D*, *H*, and *L*) resembled *ate1 ate2* double mutants (Fig. 4 *C*, *G*, and *K*), further supporting the idea that all 3 proteins act in the same pathway, and that the phenotypic alterations observed in *ate1 ate2* mutant plants are the result of impaired protein degradation by the N-end rule pathway. This interpretation is also in agreement with a recent study that showed the involvement of AtATE1/AtATE2 and PRT6 in the control of *Arabidopsis* seed germination and establishment (21).

Misexpression of *BP* in Leaves of *ate1 ate2* Double-Mutant Plants. To obtain insights into the molecular mechanisms through which R-transferases control plant development, we focused on their function during leaf formation. As described above, leaves of *ate1 ate2* double-mutant plants were serrated, mildly lobed, and wavy. Similar phenotypic alterations have been described for other *Arabidopsis* mutants, and in some of these cases the leaf margin defects correlate with misexpression of the meristem-promoting gene *BREVIPEDICELLUS* (*BP*), which belongs to the family of class I KNOTTED-like homeobox (*KNOX*) genes (24–26). To test whether *BP* is misexpressed in *ate1 ate2* double-mutant plants, we crossed a previously described *BP* reporter line (*BP:GUS*; ref. 26) into the *ate1 ate2* double-mutant background. In agreement with the known *BP* expression pattern, we detected GUS activity in the shoot apex and the hypocotyl of wild-type and *ate1 ate2* seedlings, but never in the leaves of

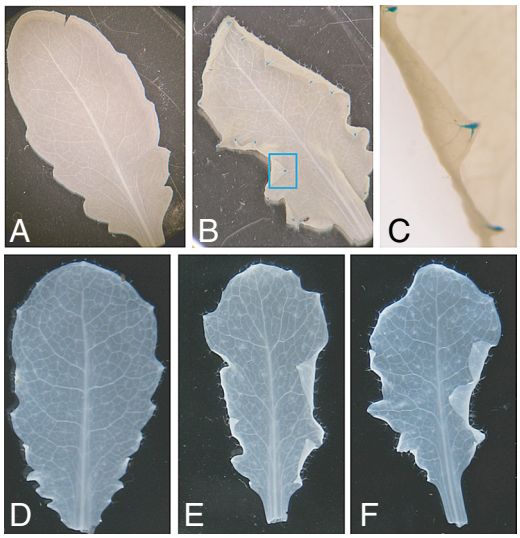

Fig. 5. *BP* is expressed in leaves of *ate1 ate2* plants but is not required for the leaf morphology defects. A *BP*::*GUS* reporter (26) was crossed into the *ate1 ate2* mutant background, and *BP* expression was monitored in plants grown in continuous light. (*A*–*C*) Whereas no *BP*::*GUS* reporter activity was detected in wild-type leaves (*A*), GUS staining was observed in the serration tips of mature *ate1 ate2* leaves (*B* and *C*). (*C*) Close-up on the leaf margin of the leaf shown in *B* (area indicated by a blue rectangle). (*D*–*F*) In contrast to *bp-1* (*D*), leaf margins of *ate1 ate2 bp-1* triple-mutant plants (*F*) are lobed and wavy, similar to those of *ate1 ate2* mutants (*E*). The leaves shown in *D*–*F* were cleared. Plants were grown in short-day conditions for 2 months.

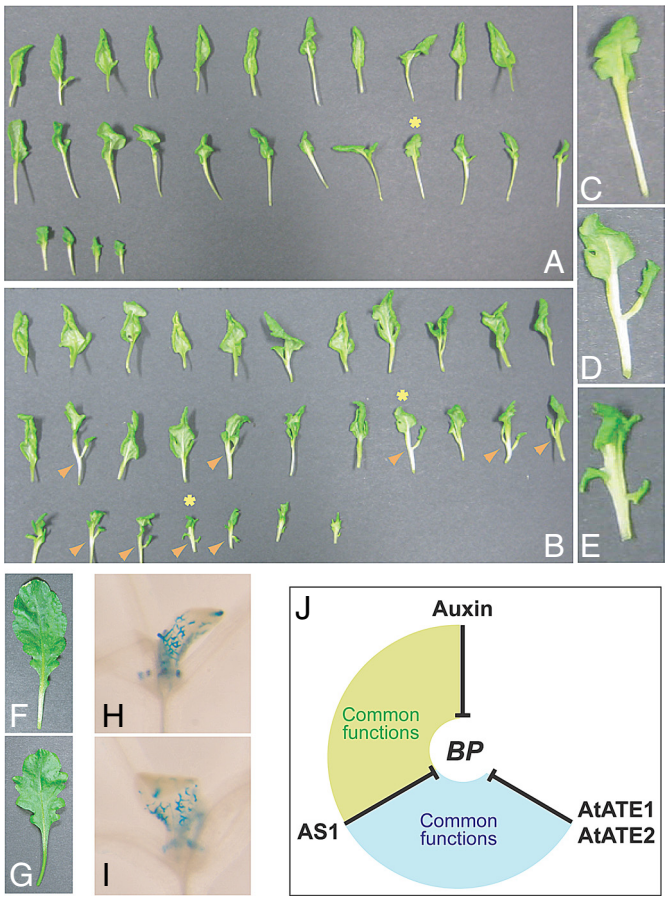

Fig. 6. R-transferases act together with *AS1*, but independently of auxin, to regulate leaf development. (*A*–*E*) Synergistic genetic interaction between *ate1 ate2* and *as1-1*. Plants were grown in short-day conditions for 2.5 months. (*A* and *B*) Leaf series of *as1-1* and *ate1 ate2 as1-1* plants, respectively, starting from leaf 15 upward. Arrowheads mark leaves with leaflets. (*C*–*E*) Close-up on leaves from the leaf series presented in *A* and *B* (indicated by asterisks), showing an *as1-1* leaf (*C*) and *ate1 ate2 as1-1* leaves with leaflets (*D* and *E*). (*E*) A late-arising leaf. (*F* and *G*) Mature leaf of an *axr1-3* single mutant (*F*) and of an *ate1 ate2 axr1-3* triple mutant (*G*), showing a more strongly lobed and wavy margin in the triple mutant. Pictures of 4-month-old plants grown in short days. (*H* and *I*) *DR5*:*GUS* reporter activity in the wild-type (*H*) and *ate1 ate2* mutant (*I*) plants. The activity of the *DR5*:*GUS* reporter was monitored by staining 5-day-old seedlings grown in long-day conditions. (*J*) A working model for the functions of R-transferases in the regulation of leaf development. AS1 and auxin act together in the control of leaf development (indicated by the green area) and repress *BP* expression (37). Misexpression of *BP* in leaves of *ate1 ate2* double-mutant plants implies that AtATE1/AtATE2 also act as negative regulators of *BP*. Moreover, the synergistic interaction between *ate1 ate2* and *as1-1* suggests that R-transferases and AS1 regulate common processes during leaf development (indicated by the blue sector). The absence of synergism between *ate1 ate2* and *axr1-3* further implies that R-transferases and auxin regulate leaf development independently.

wild-type plants (Fig. 5*A*). In contrast, in leaves of *ate1 ate2* double mutants, we observed staining at the tips of the leaf margin serrations (Fig. 5 *B* and *C*), indicating that *BP* is misexpressed in the absence of R-transferase activity. *BP* expression in serration tips has also been described for several other mutants with abnormal leaf margins, including *asymmetric leaves1* (*as1*), *auxin resistant1* (*axr1*), and the *sawtooth1/2* double mutant (24–26).

To address the role of *BP* misexpression in *ate1 ate2* plants, we constructed an *ate1 ate2 bp-1* triple mutant. Leaves of the triple mutant (Fig. 5*F*) resembled those of *ate1 ate2* double-mutant plants (Fig. 5*E*), indicating that *BP* is not essential for the formation of abnormal leaf margins in the absence of R-transferases, and that it might act redundantly with additional (*KNOX*) genes (see *Discussion*).

R-Transferases Act Redundantly with AS1 and Independently of Auxin in Leaf Development. Genes known to be involved in the repression of *BP* in *Arabidopsis* leaves include components of the AS1 transcription factor complex (27) and genes that mediate the response to auxin. Genetic evidence suggests that these pathways act in a partially redundant manner in the control of leaf development (24). To determine whether the R-transferases regulate leaf development in conjunction with one or both of these pathways, we constructed triple-mutant combinations between *ate1 ate2* and mutant alleles of genes known to be involved in the repression of *BP* in leaves. For the first of these triple mutants, we used the *AS1* allele *as1-1*. Leaves of *as1-1* mutants are asymmetric, lobed, and curl abaxially (28) (Fig. 6 *A* and *C*). In contrast, leaves of *ate1 ate2 as1-1* triple-mutant plants frequently formed 1 or 2 leaflets in the proximal region of the leaf (Fig. 6 *D* and *E*), whereas the distal part appeared to be unaffected. As in *as1-1* (26) and *ate1 ate2* mutants, the strength of these phenotypes increased with time so that late-arising leaves were more strongly affected than early-arising leaves (Fig. 6 *B*, *D*, and *E*). The appearance of leaflets in the *ate1 ate2 as1-1* triple mutant, which were not observed in either of the parental lines, suggests that the R-transferases and AS1 act in partially overlapping pathways that control leaf development.

Scanning electron microscopy carried out with leaves of *ate1 ate2* and *ate1 ate2 as1-1* plants did not reveal the presence of ectopic stipules (Fig. S5), which is a characteristic of *BP* overexpression (29). However, leaves of *as1-1* plants, in which *BP* is broadly expressed, also lack ectopic stipules (24). Therefore, other factors involved in the formation of ectopic stipules (e.g., auxin-mediated repression of *BP*; see ref. 24) appear to com-

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pensate for the loss of both AS1 and R-transferases in *ate1 ate2 as1-1* plants.

Next, we constructed a triple mutant between *ate1 ate2* and a mutant allele for *SERRATE* (*SE*), which is thought to regulate cellular competence for *KNOX* gene expression (26, 30). In contrast to the synergistic interaction observed between *se-1* and *as1-1* (26), *ate1 ate2 se-1* triple-mutant plants showed an additive phenotype (Fig. S6), which could be a consequence of the spatially limited *BP* expression domain in *ate1 ate2* leaves, in comparison with the relatively broad expression pattern of *BP* in leaves of *as1-1* plants.

To address the interplay between auxin and the R-transferases in leaf development, we constructed a triple mutant between *ate1 ate2* and a mutant allele for the *AXR1* gene. AXR1 regulates the activity of SCF-type E3 ubiquitin ligases, including that of the SCF-TIR1 auxin receptor complex (2, 31). Although AXR1 is thought to be involved in many signaling pathways (32, 33), it has been shown that the leaf defects of *axr1* mutants, which exhibit irregular and mildly lobed margins (Fig. 6*F*), are caused by a reduction in auxin response (24). Leaves of *ate1 ate2 axr1-3* triple-mutant plants were slightly more affected than those of the parental lines (Fig. 6*G* and Fig. S7*A*), but no qualitatively new phenotypes were observed. Other mutant phenotypes of *axr1-3* and *ate1 ate2* plants, such as a reduction in apical dominance, also appeared to be additive in the triple mutant (Fig. S7*B*).

Because *axr1-3* is an intermediate allele that retains some auxin response activity (31), the observed additive interaction could stem either from independent roles of R-transferases and auxin in the control of leaf development, or from a further reduction of auxin signaling in the triple mutant, owing to a loss of R-transferase function. To test the latter possibility, we first determined the effects of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) on the inhibition of root elongation in wild-type, *axr1-3*, *ate1 ate2*, and *ate1 ate2 axr1-3* seedlings. As previously described (31), *axr1-3* mutants exhibited a strongly reduced sensitivity to 2,4-D in comparison with wild-type plants (Fig. S7*C*), whereas *ate1 ate2* double mutants appeared to be slightly hypersensitive. The response of *ate1 ate2 axr1-3* plants to 2,4-D resembled that of *axr1-3* single mutants (Fig. S7*C*). These results suggest that auxin responses are not generally reduced in plants lacking R-transferases. To test auxin responses specifically in leaves, we crossed the synthetic auxin response reporter *DR5*:*GUS* (34) into the *ate1 ate2* double-mutant background. Staining of seedlings showed that the overall levels of GUS activity were similar in the wild type (Fig. 6*H*) and in *ate1 ate2* plants (Fig. 6*I*), although minor differences in the pattern of GUS activity were occasionally observed. We also measured auxin levels in *ate1 ate2* double-mutant and in wild-type plants and found no significant differences (Fig. S7*D*).

Taken together, the results of our genetic analyses suggest that R-transferases act independently of auxin but redundantly with AS1 in the control of leaf development.

Discussion

The results of the present work indicate that the R-transferases AtATE1 and AtATE2 act in a redundant manner to control diverse processes during *Arabidopsis* leaf and shoot development. We also found that the N-recognin PRT6, which acts downstream of R-transferases (Fig. 1), mediates at least some of their biological effects. Together, our findings strongly suggest that the mutant phenotypes of *ate1 ate2* plants are caused by accumulation of specific, not yet identified proteins that are normally arginylated and degraded by the N-end rule pathway.

In addition to its functions in shoot and leaf development, the arginylation branch of the *Arabidopsis* N-end rule pathway has also been shown to regulate seed germination and establishment, as well as leaf senescence (9, 21). Because only a subset of all N-end rule substrates is targeted by the arginylation branch (Fig. 1), it is likely that the functional span of the plant N-end rule pathway is comparable to that in animals and fungi. Discovering physiological substrates of the plant N-end rule pathway will be essential for a detailed understanding of its functions. Despite the broad range of known functions of the N-end rule pathway in eukaryotes and a large number of putative substrates, there are currently only ≈10 confirmed substrates (6), in part because methods for a systematic discovery of such substrates remain to be developed.

To obtain insights into the mechanisms underlying the function of R-transferases in plants, we have started to dissect their role in leaf development. We found that the *KNOX* gene *BP* is misexpressed in the leaves of *ate1 ate2* mutants, suggesting that AtATE1 and AtATE2 control leaf shape in part by repressing *BP* expression. Previous studies have shown that *KNOX* genes are main determinants of leaf shape in diverse plant species (35–37) and are required for the formation of dissected leaves (35, 37).

In the simple leaves of *Arabidopsis*, *BP* and other *KNOX* genes are repressed through the interplay of multiple pathways. The AS1 transcription factor complex plays a key role in this process (38, 39). Genetic analyses suggest that the AS1-dependent pathway acts in a partially redundant manner together with auxin to control leaf development (24). Specifically, it has been shown that *BP* is misexpressed in mutants of the auxin response gene *AXR1*. Furthermore, *axr1 as1* double mutants show synergistic interactions. Thus, auxin and AS1 functions appear to converge to repress *BP* expression in leaves (24). Notably, as in the case of *ate1 ate2* (Fig. 5 *D–F*), the removal of *BP* activity does not rescue the leaf defects of *as1* or *axr1* mutants, suggesting an involvement of additional *KNOX* genes or *KNOX*-independent pathways in the formation of abnormal leaf margins (24, 38).

To test whether R-transferases might act together with AS1 and/or auxin in the control of leaf development, we constructed *ate1 ate2 as1* and *ate1 ate2 axr1* triple mutants. Although we observed a synergistic genetic interaction between *ate1 ate2* and *as1*, *ate1 ate2* and *axr1* appeared to be additive, suggesting that R-transferases and auxin have largely nonoverlapping functions, apart from their common role in repressing *BP* expression together with AS1. This idea is supported by the different leaf phenotypes of *axr1 as1* and *ate1 ate2 as1* mutant plants. Whereas the former have deeply lobed leaf margins (24), *ate1 ate2 as1* triple mutants often form leaflets only in the proximal region of the leaf (Fig. 6 *B*, *D*, and *E*). Given the findings described above, we suggest a model (Fig. 6*J*) in which the N-end rule pathway acts independently of auxin but shares common functions with the AS1 transcription factor complex in the control of leaf development.

Materials and Methods

Plant Growth Conditions and Plant Transformation. Plants were grown on a soil–vermiculite–perlite (5:3:2) mixture at 20 °C, with cool white fluorescent light under constant illumination, in short-day (8 h of light and 16 h of darkness) or in long-day conditions (16 h of light and 8 h of darkness). *Agrobacterium*-mediated plant transformation and selection of transformants was carried out as described in *SI Materials and Methods*.

Isolation of T-DNA Insertions and Construction of Mutant Lines. The *ate1-2* (SALK_023492), *ate2-1* (SALK_040788), and *prt6-5* (SALK_051088) mutants were isolated from the SALK T-DNA collection. The presence of T-DNA insertions and mutations was verified as described in Table S1 and *SI Materials and Methods*.

In Vitro Enzymatic Assays of R-Transferases. Seven-day-old seedlings grown on 0.5 × MS plates under continuous light were used to extract proteins and measure R-transferase activity in different mutant backgrounds, as well as in the wild type. The incorporation of [^{3}H]Arg to bovine α-lactalbumin, a protein bearing N-terminal Glu, was monitored as detailed in *SI Materials and Methods*.

2,4-D and GA_3 Experiments. Surface-sterilized seeds were sowed on 0.5 × MS plates and germinated in long-day conditions. Four days after germination, seedlings were transferred to 0.5 × MS plates supplemented with 2,4-D (Sigma–Aldrich) and grown vertically for 4 days.

For GA_3 (Duchefa) treatments, plants were sprayed every 3 days with a 100 μM GA_3 solution containing 0.02% Tween-20, or with a mock solution.

Indoleacetic Acid (IAA) Content Measurement. Apices of 2-month-old short-day grown plants including young leaves (≈100 mg per sample) were collected in 100% methanol (HPLC grade; Sigma–Aldrich), and IAA was extracted as described previously (40) and in *SI Materials and Methods*.

GUS Translational Reporters and *AtATE1* and *AtATE2* Rescuing Constructs. GUS translational reporters and rescuing constructs were made as detailed in Table S2 and *SI Materials and Methods*.

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