The *Arabidopsis* aldehyde oxidase 3 (*AAO3*) gene product catalyzes the final step in abscisic acid biosynthesis in leaves

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Abscisic acid (ABA) is a plant hormone involved in seed development and germination and in responses to various environmental stresses. The last step of ABA biosynthesis involves oxidation of abscisic aldehyde, and aldehyde oxidase (EC 1.2.3.1) is thought to catalyze this reaction. An aldehyde oxidase isoform, AOδ, encoded by *AAO3*, one of four *Arabidopsis* aldehyde oxidase genes (*AAO1*, *AAO2*, *AAO3*, and *AAO4*), is the most likely candidate for the enzyme, because it can efficiently catalyze the oxidation of abscisic aldehyde to ABA. Here, we report the isolation and characterization of an ABA-deficient *Arabidopsis* mutant that maps at the *AAO3* locus. The mutant exhibits a wilty phenotype in rosette leaves, but seed dormancy is not affected. ABA levels were significantly reduced in the mutant leaves, explaining the wilty phenotype in rosettes, whereas the level in the mutant seeds was less reduced. No AOδ activity could be detected in the rosette leaves of the mutant. Sequence data showed that the mutant contains a G to A substitution in the *AAO3* gene. The mutation causes incorrect splicing of the ninth intron of *AAO3* mRNA. We thus conclude that the ABA-deficient mutant is impaired in the *AAO3* gene and that the gene product, AOδ, is an aldehyde oxidase that catalyzes the last step of ABA biosynthesis in *Arabidopsis*, specifically in rosette leaves. Other aldehyde oxidases may be involved in ABA biosynthesis in other organs.

Abscisic acid (ABA) is a plant hormone that plays an important role in many aspects of plant growth and development, including seed maturation and dormancy as well as adaptation to a variety of environmental stresses (1). The regulation of these physiological processes is caused by *de novo* synthesis of ABA. Thus, the establishment of the ABA biosynthetic pathway and the isolation of the related gene(s) are essential for determining the role of ABA.

Recently, genes encoding ABA biosynthetic enzymes have been cloned, which has led to a better understanding of the regulation of ABA biosynthesis (2–4). The most important advances were the isolation of a gene for zeaxanthin epoxidase (ZEP), using the *Nicotiana plumbaginifolia* mutant *aba2* (5) and the isolation of the 9-*cis*-epoxycarotenoid dioxygenase (NCED) gene, using the maize mutant *vp14* (6, 7). ZEP converts zeaxanthin to violaxanthin by a two-step epoxidation, and NCED catalyzes the oxidative cleavage of 9-*cis*-xanthophylls to form xanthoxin. *NCED* cDNAs also were cloned from *Phaseolus* (8), tomato (9), and cowpea (10). It was shown that *NCED*, but not *ZEP*, expression is up-regulated in water-stressed leaves, indicating a regulatory role for NCED in ABA biosynthesis. It was proposed that there is a *NCED* gene family in plants and therefore the different *NCED* genes might be responsible for regulation of ABA biosynthesis in different tissues and under different environmental conditions (8).

The recent findings established that zeaxanthin epoxidase and NCED are located in plastids and the product of the NCED reaction, xanthoxin, is converted to ABA in the cytosol by two oxidation steps, via abscisic aldehyde (4). Several mutants related to the final step of ABA biosynthesis, the oxidation of abscisic aldehyde to ABA, have been isolated. These include the *Arabidopsis aba3* mutant, the *aba1* mutant of *N. plumbaginifolia*, and tomato *flacca* and *sitiens* mutants. However, these mutants, except for *sitiens,* are not impaired in abscisic aldehyde oxidase, but are deficient in the synthesis of the molybdenum cofactor (Moco), which is necessary for aldehyde oxidase activity (11–15). Only the *sitiens* mutant of tomato is thought to have a mutation in a structural gene of an aldehyde oxidase specific for abscisic aldehyde (11, 16). Although three putative aldehyde oxidase cDNAs have been cloned from tomato (17, 18), the corresponding gene for *sitiens* has not yet been identified.

The existence of aldehyde oxidase isoforms has been reported in several plants (17, 19–22). Our previous work demonstrated the presence of three aldehyde oxidase isoforms, AOα, AOβ, and AOγ, in extracts of *Arabidopsis* seedlings by activity staining after native PAGE (21). They had relatively wide substrate specificity, but abscisic aldehyde was a poor substrate for all three. Subsequently, we cloned four *Arabidopsis* aldehyde oxidase cDNAs (*AAO1, AAO2*, *AAO3*, and *AAO4*, formerly called *atAO-1*, *atAO-2, atAO-3*, and *atAO-4*, respectively) (23). *AtAO1*, *AtAO2*, and *AtAO3*, corresponding to *AAO1*, *AAO4*, and *AAO2*, respectively, also were cloned independently by Hoff *et al.* (24). We also found an aldehyde oxidase isoform, AOδ, which is encoded by *AAO3*. This isoform efficiently oxidizes abscisic aldehyde to ABA in rosette leaves. *AAO3* mRNA was abundant in the rosette leaves, and the expression was up-regulated by dehydration (25). However, our preliminary experiments showed no significant increase in enzyme activity and AAO3 protein level after desiccation, indicating that the oxidation of abscisic aldehyde is not a limiting step in ABA biosynthesis in leaves as already shown by Sindhu and Walton (26). In this study, we describe an ABA-deficient mutant of *Arabidopsis* defective in the *AAO3* gene. Characterization of the mutant demonstrated that AOδ is an abscisic aldehyde oxidase that is predominantly involved in ABA biosynthesis in leaves.

Abbreviations: ABA, abscisic acid; Moco, molybdenum cofactor; NCED, 9-*cis*-epoxycarotenoid dioxygenase; RT-PCR, reverse transcription–PCR; L*er*, Landsberg *erecta*.

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Materials and Methods

Mutant Isolation and Genetic Characterization. Seeds of the *abi3–1* mutant [in Landsberg *erecta* (L*er*) genetic background] were imbibed in a solution of 15 mM ethyl methanesulfonate. The M_2 population that was described before in Ooms *et al.* (27) was screened for wilty plants. Plant growth and germination experiments were performed as described by Léon-Kloosterziel *et al.* (28). Water loss was determined by weighing well-watered plants that had just started to bolt. The rosettes were cut from their roots, placed on filter paper on a bench at ambient temperature, and weighed every half-hour.

Mapping was performed on F_3 lines derived from the cross of the *aao3* mutant with wild-type Columbia that were phenotypically classified as having a slightly wilty or wild-type phenotype. F_3 lines segregating for the mutation were not included in the analysis. Cosegregation of the mutant and the *AAO3* gene was tested with the cleaved amplified polymorphic sequence (CAPs) marker described by Sekimoto *et al.* (23).

ABA Determinations. Plant material was grown as before (29) and used for ABA determinations when still in the rosette stage. ABA was extracted and purified as described (30). Quantification of methyl-ABA was performed with a Hewlett–Packard 6890 gas chromatograph, equipped with an electron capture detector, and using endrin as an internal standard.

Enzyme Extraction and Activity Stain. For activity determination, Northern blotting, and Western blotting, *Arabidopsis thaliana* L*er* wild-type and mutant seeds were sown in pots containing vermiculite and watered with nutrient solution under 16 h light and 8 h darkness at 22°C for about 2 months. Enzyme extraction and activity staining after native PAGE were carried out essentially according to the methods described (20). Plant tissue was homogenized in 8 ml/g fresh weight (FW) extraction buffer [50 mM Tris·HCl, pH 7.5/1 mM EDTA/1 μM sodium molybdate/10 μM FAD/2 mM DTT/one tablet/100 ml protease inhibitor (Complete Protease inhibitor mixture tablets, Roche Diagnostics) and Polyclar AT (0.2 g/g FW)]. The extract was fractionated with ammonium sulfate (0–60% saturation), and excess protein was removed by heat treatment (3 min at 60°C). After electrophoresis with 7.5% native acrylamide gel at 4°C, activity bands of aldehyde oxidase were developed with aldehyde substrate (200 μM) at 30°C in the dark for 30–60 min.

Sequencing of the *AAO3* Gene. Genomic DNA was isolated from L*er* and the mutant using Plant DNA ZOL Reagent (GIBCO/BRL) according to the manufacturer's instructions. Based on the sequence of *AAO3* gene from Columbia background (AC007154) reported by the genome project (http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?uid=AC007154&form=6&db=n&Dopt=gDopt=g), primers 5′-TGTGTATGTTGATACAAGAGAGT-3′ and 5′-GGTTTTGAAACCATTAGTTATGC-3′, corresponding to nucleotides 2453–2475 and 10258–10280 of AC007154, respectively, were designed to amplify 7.8 kb of the *AAO3* genomic region, including 2.6 kb of the 5′ upstream sequences. The PCR fragments were cloned into the pCR2.1 vector (Invitrogen), and several independent clones were sequenced and compared between L*er* and the mutant in three independent experiments. The wild-type *AAO3* clone was used for complementation of the mutant.

RNA Extraction, Reverse Transcription–PCR (RT-PCR), and Northern Blotting. Total RNA was extracted by the method described by Verwoerd *et al.* (31), except that the buffer composition was 100 mM Tris·HCl, pH 8.0/100 mM LiCl/10 mM EDTA/1% SDS. For RT-PCR, first-strand cDNA was synthesized by using the superscript preamplification system (GIBCO/BRL). PCR was performed by using oligonucleotide primers 5′-TACACTAGGTATGATCCAAGGAG-3′ and 5′-ACACTATACAATCCGCAAAGAGA-3′, designed at the positions corresponding to nucleotides 3423–3445 and 4138–4160 of *AAO3* cDNA (AB016622), respectively, to amplify a fragment of 736-bp wild-type *AAO3* cDNA. Northern blotting was carried out by using 10 μg total RNA. RNA samples were loaded on a 1.5% agarose gel containing formaldehyde and Mops [3-(*N*-morpholino)propanesulfonic acid] buffer and transferred to a nylon membrane. Hybridization was performed by using the ^{32}P-dCTP-labeled *AAO3* full-length cDNA and a 367-bp cDNA fragment of *APETALA2* (*AP2*) digested with *Bam*HI and *Xho*I as probes under highly stringent conditions (32). No cross-hybridization occurred, because the probe showed a specific pattern of the hybridization between four *AAO* probes (25).

Table 1. ABA levels (μg/g dry weight) in rosettes, siliques, and seeds of wild type and two ABA-deficient mutants

Genotype	Turgid rosettes	Wilted rosettes	Immature siliques	Mature dry seeds
Wild type (L*er*)	0.35	2.20	0.59	0.24
aao3	0.13	0.46	0.25	0.14
aba3-2	nd	nd	0.10	0.03

nd = not determined. In earlier measurements (28) stressed leaves of *aba3-2* contained only 8% of the ABA content in L*er* stressed rosettes. All measurements were repeated once with similar results.

Western Blotting. Anti-AAO3 antibodies were obtained as described (25). Western blotting was performed by using a Vectastain Elite ABC kit (Vector Laboratories) diluted 100-fold in TBS (20 mM Tris·HCl, pH 7.5/150 mM NaCl) containing 0.05% Tween-20 according to the previous study (20). Peroxidase activity was visualized by staining with an immunostain HRP-1000 kit (Konika, Tokyo).

Complementation. *AAO3* genomic DNA obtained as described above was excised and cloned into transformation vector pPZP211 (33). Transformation of the mutant was performed according to the procedure described by Bechtold *et al.* (34).

Results

Isolation of a Wilty Mutant, *aao3*, of *Arabidopsis*. Screening of M_2 plants derived from ethyl methanesulfonate mutagenesis of the *abi3–1* mutant under greenhouse conditions led to the identification of a mutant with a mild wilty phenotype. This mutant, named *aao3* for reasons explained hereafter, was backcrossed to the L*er* wild type and in the segregating progeny, F_3 lines with a wilty phenotype, but lacking the *abi3–1* mutation, were identified. ABA determinations of rosettes (Table 1) indicated that this mutant has a reduced ABA content. Seed germination experiments with the homozygous wilty mutant indicated that, in contrast to all known wilty ABA-deficient and ABA-insensitive mutants in *Arabidopsis* (35), the seeds had dormancy comparable to that of L*er*. This finding suggested that the mutant represented a novel locus affected in ABA biosynthesis or catabolism. This was further confirmed by the complementation of the wilty phenotype in F_1 hybrids derived from crossing the mutant to the ABA-deficient mutants, *aba1*, *aba2*, and *aba3* (data not shown).

ABA Levels in Wild-Type and Mutant Plants. ABA levels were determined in turgid and water-stressed rosette leaves, immature siliques, and mature dry seeds (Table 1). In the turgid rosettes of the *aao3* mutant, the ABA level was about one-third of that in wild type and the increase after water stress was much less than in wild-type rosettes. In siliques and dry seeds of *aao3* plants, the

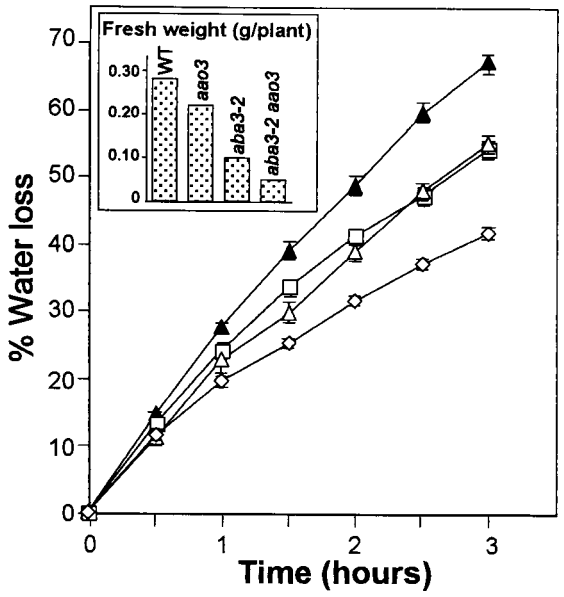

Fig. 1. Water loss of wild type (◇), *aba3–2* (△), *aao3* (□), and *aba3–2 aao3* (▲) expressed as loss of fresh weight. (*Inset*) The average fresh weight of the genotypes is shown. WT, wild type.

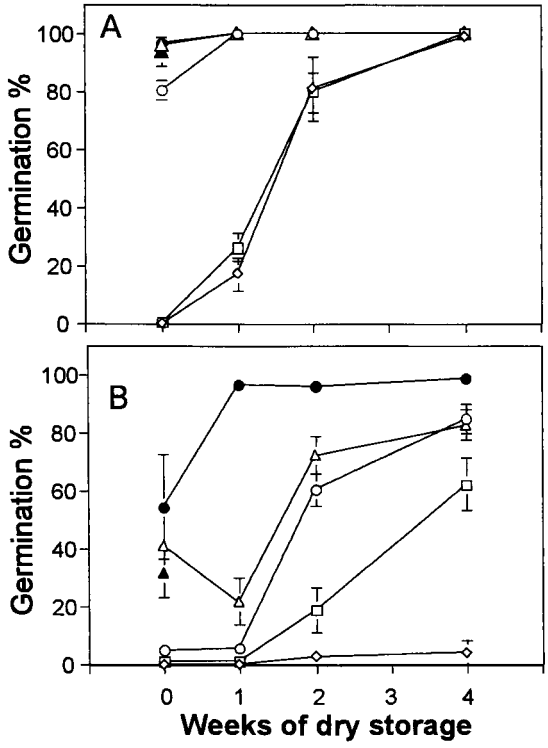

Fig. 2. Germination percentage of wild type (◇), *aba3–2* (△), *aao3* (□), *abi3–1* (○), *aba3–2 aao3* (▲), and *abi3–1 aao3* (●) seeds in the light (*A*) and darkness (*B*).

amount of ABA was less reduced (about one-half of wild type) compared with the reduction in rosettes. Immature siliques and dry seeds of *aba3–2* mutant, deficient in Moco that is required for aldehyde oxidase activity (13), contained a more reduced amount of ABA (1/6 to 1/10 of wild type).

Characterization of *aao3* Mutant Phenotype. The mutant was characterized physiologically in comparison to the previously described *aba3–2* mutant (28) and the double mutants of *aao3* with *abi3–1* and *aba3–2* by (*i*) determination of water loss in detached rosettes, and (*ii*) measurement of the loss of seed dormancy caused by after-ripening.

The results in Fig. 1 indicate that the enhanced water loss characteristics of *aao3* are comparable to those of the *aba3–2* mutant, which has a smaller vegetative mass than *aao3.* However, plants of the double mutant *aba3–2 aao3* have a very weak growth habit, which correlates with a much more rapid water loss by detached rosettes. Results of germination experiments (Fig. 2*A*) confirmed that seeds of the *aao3* mutant have dormancy similar to that of L*er* seeds when tested in white light. However, when tested in darkness, *aao3* seeds germinated slightly better than wild-type seeds (Fig. 2*B*). Some further reduction in dormancy over that of *abi3–1* was observed in the double mutant, *abi3–1 aao3*. However, the double mutant did not have the green seed phenotype observed for the *aba3–abi3* double mutant (28). This almost normal phenotype for dormancy in light coincided with a reduction in ABA content both during seed development and in mature seeds (Table 1). However, the reduction in ABA levels observed in these tissues was less than in leaves.

Mapping of the Mutant. Mapping of the mutant revealed that the gene was located on chromosome 2, very close to the *erecta* mutation, where no previously known ABA-deficient mutants had been mapped. This position is similar to that of *AAO3,* which was shown to encode an abscisic aldehyde oxidase expressed in *Arabidopsis* leaves (25). The nearly full-length *AAO3* cDNA (AB016622) exhibits a 198-nt 5′ untranslated region, followed by a 3,999-nt ORF and a 121-nt 3′ untranslated sequence. The ORF of the cDNA predicts a protein of 1,332 aa with molecular weight of 146,665, and the protein contains the conserved sequence for two iron-sulfur centers and five motifs involved in Moco binding. Colocation of the mutant and *AAO3* polymorphism described before (23) was confirmed by testing 22 homozygous mutant and 13 homozygous wild-type F_3 lines derived from the cross, *aao3* × Columbia. In all 35 lines *aao3* cosegregated with the morphological *erecta* marker. This finding is in agreement with the location of *AAO3*, 378 kbp south of *erecta* (data obtained from the TIGR database; http://www.tigr.org/tdb/ath1/htmls/).

Abscisic Aldehyde Oxidase in the Mutant and Wild-Type Plants. We next investigated activities of aldehyde oxidase, including the AOδ isoform encoded by *AAO3*, both in wild-type and *aao3* plants. The activity also was checked in *aba3–2* plants, a mutant lacking all aldehyde oxidase activities because of its Moco deficiency (Fig. 3). In rosette leaves of wild-type plants, an

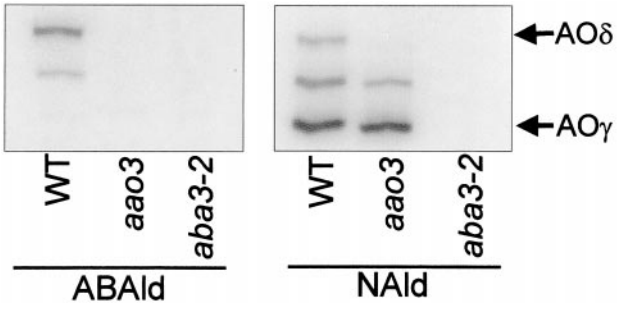

Fig. 3. Aldehyde oxidase activities in the rosette leaves of *aao3*, *aba3–2*, and wild-type (WT) *Arabidopsis*. Enzyme extracts were subjected to native PAGE, and activity bands were developed by using abscisic aldehyde (ABAld) or 1-naphthaldehyde (NAld). Each lane was loaded with 65 μg of protein.

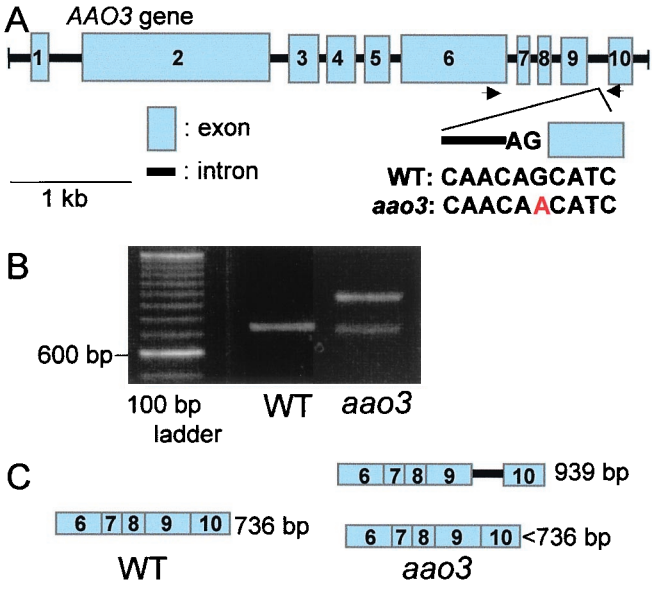

Fig. 4. Structure and expression of *AAO3* and *aao3*. (*A*) Structure of the *AAO3* gene and location of the mutation at the end of the ninth intron. Arrows indicate the position where primers for RT-PCR were designed. WT, wild type. (*B*) RT-PCR fragments obtained from cDNAs synthesized from total RNA prepared from rosette leaves using primers represented in *A*. (*C*) Illustration of RT-PCR fragments detected in wild type and *aao3*.

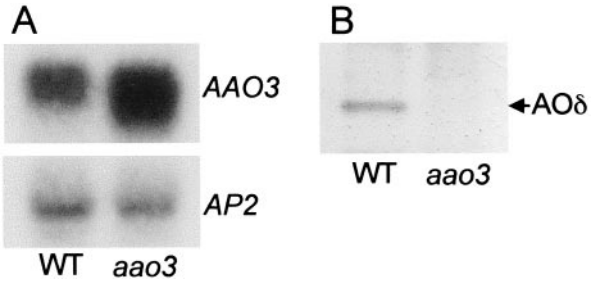

Fig. 5. Expression of *AAO3* mRNA and protein in the rosettes of wild type (WT) and the *aao3* mutant. (*A*) Total RNA was extracted from rosettes of 2-month-old plants. Ten micrograms of RNA was separated on 1.5% (wt/vol) agarose gels containing 5% (vol/vol) formaldehyde. Specific probes for *AAO3* and *AP2* were used for hybridization. (*B*) Immunoblotting with protein extracts from the rosette leaves of wild-type and *aao3* plants. Crude enzyme extracts (60 μg protein) of rosettes were subjected to native PAGE followed by immunoblot analysis using anti-AAO3 antibodies. Arrow indicates the position of AOδ.

intense band of AOδ was detected with the abscisic aldehyde substrate. In contrast, the *aao3* and *aba3–2* mutants lacked the activity. When 1-naphthaldehyde was used as a substrate, the *aao3* mutant exhibited two activity bands other than AOδ that also were detected in wild type, whereas the *aba3–2* mutant lacked all activities. This indicated that *aao3* is specifically impaired in the activity of AOδ. Almost no abscisic aldehyde oxidase activity was detected in siliques, dry seeds, and imbibed seeds of all three genotypes (data not shown). The band observed at the middle position in Fig. 3 probably represents AOβ, a heterodimer of AAO1 and AAO2 (36), and/or another isoform originating as a heterodimer consisting of AAO2 and AAO3 products as discussed by Seo *et al.* (25).

Detection of a Mutation in *AAO3* in the *aao3* Mutant. To determine whether the *aao3* mutant contains a mutation in the *AAO3* gene, genomic DNAs of *AAO3* from L*er* wild type and the mutant were sequenced. Six differences in the nucleotide sequences were found between the *AAO3* genes from Columbia and L*er* backgrounds. The differences between Columbia and L*er* in their sequences are as follows; A and C, C and G, T and G, G and T, T and C, and T and G at nucleotide positions 5127, 5412, 5429, 5917, 6133, and 6803 of the Columbia *AAO3* gene (AC007154). In the *aao3* mutant, a single base pair substitution was found at the end of the ninth intron of the *AAO3* gene (Fig. 4*A*). The mutation causes a transition of the conserved 3′ splice site AG to AA. It is expected that the mutation results in incorrect splicing of the intron. RT-PCR was performed by using total RNA prepared from rosettes of wild type and *aao3* mutant (Fig. 4*B*). Primers were designed to amplify 736-bp *AAO3* cDNA fragments of wild type including the region where the ninth intron had been spliced out. A cDNA fragment of the correct size was detected only in wild-type rosette leaves. cDNA fragments of two different sizes were found in *aao3* leaves. One fragment had a size of around 950 bp, which corresponds to the size of the 736-bp fragment plus the 203 bp of the ninth intron. The other was slightly smaller than the 736-bp fragment (Fig. 4*C*). The RT-PCR fragments obtained from *aao3* mutant RNA were cloned into the sequencing vector, and three independent clones of the longer fragment and 13 clones of the shorter fragments than wild-type DNA were sequenced. The longer fragments contained the entire ninth intron in their sequence. Three different sequences were detected among the shorter fragments. Incorrect splicing had resulted in shorter transcripts by 2, 6, or 30 bp compared with wild type and no correctly spliced sequence was detected. The incorrect splicing caused frameshifts, or deletion of amino acids. These frameshifts or deletions occurred in one of the Moco binding sites "SGEPPL" in the *AAO3* gene (23). The results indicate that there are hardly any correct mRNA transcripts in *aao3* plants and that, if the translation from this incorrectly spliced mRNA proceeds to some extent, the protein cannot effectively oxidize abscisic aldehyde to ABA.

Expression of *AAO3* in Rosette Leaves. Expression of *AAO3* mRNA and AAO3 protein in the rosette leaves of wild type and *aao3* mutant were analyzed by Northern and Western blotting, respectively. *AAO3* transcripts were detected both in the wild type and *aao3* mutants. However, a more intense signal was observed in the mutants, whereas *AP2* mRNA expressed ubiquitously in plant organs (37) was detected at the same level in both wild-type and mutant leaves (Fig. 5*A*). Western blotting, using anti-AAO3 antibodies, revealed that AOδ is not present in detectable amounts in extract from leaves of mutant plants (Fig. 5*B*).

Complementation of the *aao3* Mutant by the *AAO3* Gene. The *AAO3* gene was introduced into *aao3* mutant plants by *Agrobacterium*-mediated transformation. Transformants were selected for kanamycin resistance, and a T_3 population that was homozygous for the transgene was obtained. Leaf extracts from these transgenic plants exhibited the activity of AOδ (Fig. 6*A*), and the wilty phenotype of *aao3* mutant plants was restored to normal (Fig. 6*B*). We conclude from these results that the mutation in *AAO3* causes the ABA deficiency of the mutant and that AOδ encoded by AAO3 is responsible for the last step in ABA biosynthesis in rosette leaves of *Arabidopsis*.

Discussion

Aldehyde oxidase is involved in ABA biosynthesis by catalyzing the last step of the pathway. Our recent results revealed that in *Arabidopsis* there is a gene family consisting of at least four aldehyde oxidase genes (23) and that one of the genes, *AAO3*, encodes AOδ, which has a high specificity for abscisic aldehyde (25). The *AAO3* mRNA is expressed mainly in rosette leaves, and most of the AOδ enzyme activity is detected in leaf tissues. Thus,

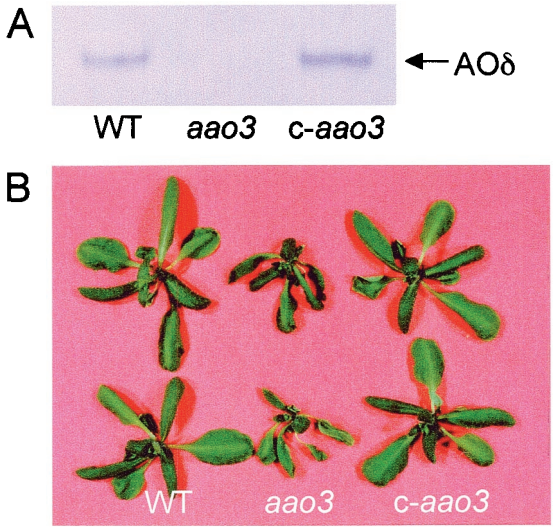

Fig. 6. Complementation of *aao3* mutant. (*A*) Activity of AOδ in wild type (WT), *aao3*, and *aao3* complemented with wild-type *AAO3* gene (c-*aao3*) was developed by using abscisic aldehyde as a substrate. Each lane was loaded with 60 μg of protein. (*B*) Plants of wild type, *aao3*, and c-*aao3* were cut from roots and left for 1 h at room conditions.

we proposed that AOδ is involved in ABA biosynthesis in *Arabidopsis* leaves and that other aldehyde oxidases are involved in ABA biosynthesis in other organs, such as roots, siliques, and seeds.

The present study shows that the ABA deficiency in *aao3* is caused by a mutation in the *AAO3* gene. The mutant exhibits a wilty phenotype in the leaves, but is not or hardly affected in seed dormancy. In fact, the ABA level is reduced in rosettes of *aao3* compared with wild type, but a less-reduced ABA level is observed in siliques and seeds of *aao3*. Although a significantly reduced ABA level in siliques and seeds was observed, one explanation for the lack of a nondormant phenotype might be that the ABA levels in the *aao3* mutant are above the threshold required for induction of dormancy. These observations lead to the following question: How is ABA synthesized in seeds of the *aao3* mutant? One possibility is that the *aao3* mutant is leaky. However, in leaky *aba1* mutants of *Arabidopsis*, such as *aba1–3*, the reduction in ABA content is similar in seeds and leaves, and a clear nondormant phenotype is observed (38, 39). Furthermore, no detectable amounts of correctly transcribed mRNA, AOδ protein, and its activity were observed in *aao3* rosette leaves (Figs. 3–5). Thus, it is unlikely that the ABA present in the *aao3* mutant is synthesized by the remaining activity of AOδ. A second possibility is that other aldehyde oxidase(s) is able to oxidize abscisic aldehyde to produce ABA in this mutant. *AAO4* is a good candidate for a gene encoding an enzyme, which is able to oxidize abscisic aldehyde, because its transcript is expressed mainly in siliques (25). However, the enzymatic nature of the AAO4 protein has not yet been determined.

As shown in Table 1, a significant residual level of ABA (37% and 20%) was detected in turgid and wilted rosettes of the *aao3* mutant, respectively, even though no abscisic aldehyde oxidase activity was detected in the leaves. It is possible that ABA in the leaves of the *aao3* mutant is imported from other organs, such as the roots. It also could be produced by other aldehyde oxidase isoforms and/or via the shunt pathway (40).

In tomato, abscisic aldehyde oxidase is most likely encoded by the *Sitiens* (*Sit*$^+$) gene (11, 16). Plants of the *sitiens* (*sit*) mutant show more extreme symptoms of ABA deficiency and reduced growth than the *flacca* (*flc*) and *notabilis* (*not*) mutants because of excessive water loss (41). Thus, the severe phenotype of the *sit* mutant differs from the mild symptoms observed in the *aao3* mutant. The two mutants also differ in that seed dormancy is not or hardly affected in *aao3*, whereas *sit* mutants are clearly nondormant (42). Because the *sit* mutant in tomato is more extreme it is possible that the *Sit*$^+$-encoded aldehyde oxidase alone is responsible for the last step in ABA biosynthesis in all tissues.

Aldehyde oxidases require a Moco to which sulfur is added. Previously it was shown that the addition of sulfur is mediated by the *ABA3* gene product in *Arabidopsis* (13). An additive effect for water loss in the *aba3–2* and *aao3* double mutant (Fig. 1) can be explained by the finding that the *aao3* mutant is impaired only in the AOδ isoform, whereas the *aba3–2* mutant, which itself has a "leaky" ABA deficiency (28), is impaired in all aldehyde oxidase isoforms as well. Water loss from excised rosettes of *aba3–2* and *aao3* was very similar, although plant vigor was much more reduced in the *aba3–2* than in the *aao3* mutant. This might be because ABA deficiency in *aao3* is less extreme at this later stage of rosette development than at early stages, or it may be because of the pleiotropic effects of the Moco deficiency in the *aba3–2* mutant.

The present data indicate that AOδ, the *AAO3* product, catalyzes the last step of ABA biosynthesis only in rosette leaves. Transgenic lines, such as knockout null mutants and overexpression lines for each *AAO* gene, will be useful to elucidate the roles of the individual *AAO* genes in ABA biosynthesis in relation to plant development and environmental responses.

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