Two New Alleles of the *abscisic aldehyde oxidase 3* Gene Reveal Its Role in Abscisic Acid Biosynthesis in Seeds[1]

Miguel González-Guzmán, David Abia, Julio Salinas, Ramón Serrano, and Pedro L. Rodríguez*

Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-Consejo Superior de Investigaciones Científicas, Camino de Vera, E–46022 Valencia, Spain (M.G.-G., R.S., P.L.R.); and Departamento de Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Carretera de la Coruña, E–28040 Madrid, Spain (D.A., J.S.)

The abscisic aldehyde oxidase 3 (AAO3) gene product of Arabidopsis catalyzes the final step in abscisic acid (ABA) biosynthesis. An *aao3-1* mutant in a Landsberg *erecta* genetic background exhibited a wilty phenotype in rosette leaves, whereas seed dormancy was not affected (Seo et al., 2000a). Therefore, it was speculated that a different aldehyde oxidase would be the major contributor to ABA biosynthesis in seeds (Seo et al., 2000a). Through a screening based on germination under high-salt concentration, we isolated two mutants in a Columbia genetic background, initially named *sre2-1* and *sre2-2* (for salt resistant). Complementation tests with different ABA-deficient mutants indicated that *sre2-1* and *sre2-2* mutants were allelic to *aao3-1*, and therefore they were renamed as *aao3-2* and *aao3-3*, respectively. Indeed, molecular characterization of the *aao3-2* mutant revealed a T-DNA insertional mutation that abolished the transcription of *AAO3* gene, while sequence analysis of *AAO3* in *aao3-3* mutant revealed a deletion of three nucleotides and several missense mutations. Physiological characterization of *aao3-2* and *aao3-3* mutants revealed a wilty phenotype and osmotolerance in germination assays. In contrast to *aao3-1*, both *aao3-2* and *aao3-3* mutants showed a reduced dormancy. Accordingly, ABA levels were reduced in dry seeds and rosette leaves of both *aao3-2* and *aao3-3*. Taken together, these results indicate that AAO3 gene product plays a major role in seed ABA biosynthesis.

Abscisic acid (ABA) plays a major role in seed development, adaptive plant responses to water deprivation, and sugar sensing (Cheng et al., 2002; Finkelstein et al., 2002). The level of ABA increases in plants during seed development and under environmental stresses, particularly drought and salinity (Finkelstein and Rock, 2002). During seed development, ABA levels rise at the end of embryogenesis, are maximal during mid development when storage reserves are accumulated, and then decline during desiccation (Karssen et al., 1983). During embryogenesis and seed formation, ABA is implicated in the control of many events such as embryo morphogenesis, storage protein synthesis, desiccation tolerance, as well as the onset and maintenance of dormancy (Bentsink and Koornneef, 2002). Recently, the importance of ABA to prevent seed germination and to promote postgermination developmental arrest under unfavorable water conditions has been genetically documented (Lopez-Molina et al., 2001; Gonzalez-Guzman et al., 2002). Later on, the increase of ABA levels in response to salinity and drought stress is a crucial adaptation to cope with these stresses, which has a wide impact on regulation of gene expression (Hoth et al., 2002; Seki et al., 2002).

Recently, all the major genes encoding the enzymes that catalyze the different steps of ABA biosynthesis have been identified and the biosynthetic pathway mostly elucidated (Schwartz et al., 2003; Xiong and Zhu, 2003). Thus, the biosynthesis of ABA can be traced from the epoxidation of zeaxanthin by zeaxanthin epoxidase, which leads to all-trans-violaxanthin (Marin et al., 1996). Next, this compound is converted in 9-cis-violaxanthin and 9-cis-neoxanthin through an uncharacterized mechanism. Nine-cis-epoxycarotenoids suffer an oxidative cleavage catalyzed by nine-cis-epoxicarotenoid dioxigenase (NCED), leading to production of xanthoxin (Schwartz et al., 1997). Then, xanthoxin is converted to abscisic aldehyde by a short-chain alcohol dehydrogenase, which is a NAD-dependent enzyme (Cheng et al., 2002; Gonzalez-Guzman et al., 2002). Finally, abscisic aldehyde is oxidized to ABA by AAO3 (abscisic aldehyde oxidase; Seo et al., 2000a), which requires a molybdenum cofactor (MoCo) that is synthesized by the MoCo sulfurase ABA3 (Bittner et al., 2001; Xiong et al., 2001).

While the main features of the pathway of ABA biosynthesis have been elucidated during the last years, the regulatory mechanisms of the biosynthetic genes remain largely unknown at the molecular level. Additionally, some aspects of ABA biosynthesis still

are not well characterized. For instance, the formation of the 9-cis isomers that are cleaved by NCED has not been clearly established (Schwartz et al., 2003). Another point that deserves further research is the biosynthesis of ABA in seeds. Thus, while it has been demonstrated that AAO3 is responsible for ABA biosynthesis in leaves, according to the phenotypic characterization of the *aao3-1* allele, it has been suggested that a different aldehyde oxidase catalyzes the last step of ABA biosynthesis in seeds (Seo et al., 2000a). For instance, *aao3-1* seeds (in a Landsberg *erecta* [L*er*] genetic background) do not show a clear change in dormancy (Seo et al., 2000a), whereas other known ABA-deficient mutants have much less dormant seeds. Additionally, Seo et al. (2000b) failed to detect *AAO3* gene expression in siliques and dry seeds.

Our understanding of the pathway of ABA biosynthesis (and by extension of ABA functions) has been greatly aided by the identification and characterization of ABA-deficient mutants. These mutants have been identified on the basis of their wilty phenotype or seed germination characteristics (Koornneef et al., 1982; Leon-Kloosterziel et al., 1996). Particularly, mutants able to germinate and carry out early growth in medium containing a high-NaCl concentration (*sre* mutants for salt resistant) were found to be allelic to ABA-deficient mutants (Gonzalez-Guzman et al., 2002). Thus, the *sre1* mutant was found to be allelic to *aba2-1* mutant (Gonzalez-Guzman et al., 2002). In this work, we report the characterization of two new *sre* mutants in a Columbia (Col) genetic background, *sre2-1* and *sre2-2*, which were found to be allelic to *aao3-1*. These new alleles of *AAO3*, in addition to a wilty phenotype, show reduced dormancy, paclobutrazol- and osmotic stress-resistant germination, as well as reduced ABA levels in seeds, indicating that the AAO3 gene product plays an important role in ABA biosynthesis in seeds.

RESULTS

Identification and Physiological Characterization of New AAO3 Alleles

During the course of a screening for mutants able to germinate and develop green cotyledons under inhibitory concentrations of NaCl, we identified four complementation groups, which were named *sre1* to *sre4*, in a germination assay (Gonzalez-Guzman et al., 2002). The *sre2* mutant was able to bypass the developmental arrest induced by high-osmoticum, that is, 200 mM NaCl or 400 mM mannitol (Fig. 1A). Two allelic mutants (in a Col genetic background), *sre2-1* and *sre2-2*, were identified for this locus (Table I). Genetic analysis indicated that the mutant phenotype was caused by a single recessive mutation (Table I). ABA promotes the inhibition of both seed germination and early seedling development under low-water-potential

conditions. Therefore, screenings for mutants able to bypass the ABA-mediated blockage of germination and development under osmotic stress usually result in the identification of ABA-deficient mutants (Leon-Kloosterziel et al., 1996; Quesada et al., 2000; Gonzalez-Guzman et al., 2002). Indeed, complementation analyses of both *sre2-1* and *sre2-2* showed that they were allelic to *aao3-1* (Table I), which is the only mutant allele described currently for the *AAO3* gene. Therefore, we renamed *sre2-1* and *sre2-2* mutants as *aao3-2* and *aao3-3*, respectively. The *aao3-1* allele was identified in a mutant showing a mild wilty phenotype under greenhouse conditions. Transpiration assays of *aao3-2* and *aao3-3* mutants also indicated enhanced water loss as compared to wild type (Fig. 1B).

As both *aao3-2* and *aao3-3* were isolated in a screening based on seed germination under high-osmoticum, we presumed that they could have reduced ABA levels in seeds. To experimentally support this hypothesis, we measured ABA levels both in dry seeds and seeds imbibed and incubated for 24 h in 200 mM NaCl (Table II). ABA levels in dry seeds of *aao3-2* and *aao3-3* were approximately 35% of wild type. Interestingly, upon seed imbibition and incubation in 200 mM NaCl, ABA levels were still approximately 3-fold lower in *aao3-2* and *aao3-3* than in wild type. In addition to the phenotypes observed in seeds of *aao3-2* and *aao3-3* mutants, we also noticed a wilty phenotype under low humidity conditions (Fig. 1B). Therefore, we also measured ABA levels in rosette leaves of both *aao3-2* and *aao3-3* under unstressed or water-stress conditions (Table II). Rosette leaves of *aao3-2* and *aao3-3* mutants contained less ABA than wild type, and ABA levels upon water stress were notably lower in the mutants compared to wild type.

Molecular Characterization of the *aao3-2* and *aao3-3* Alleles

The *aao3-2* and *aao3-3* mutants were isolated from a seed population mutagenized with T-DNA. Therefore, we analyzed whether the mutant phenotypes were linked to a T-DNA insertion. Homozygous *aao3-2* plants were crossed to Col wild-type plants. From the segregating F$_2$ generation, homozygous *aao3-2* individuals were selected and scored for the presence of the T-DNA by Southern blot analysis. The analysis of 88 F$_2$ chromosomes revealed cosegregation of the salt-resistant phenotype and the presence of the T-DNA (data not shown). Plant T-DNA flanking sequences were isolated by plasmid rescue, and sequence analysis revealed that the T-DNA was inserted at nucleotide 282 of *AAO3* (Fig. 2A). The T-DNA insertion in the *aao3-2* allele abolished the expression of the *AAO3* gene, as detected by northern blot analysis (Fig. 2B).

A similar analysis to the one described above failed to show cosegregation between the salt-resistant phenotype of *aao3-3* mutant and a T-DNA insertion.
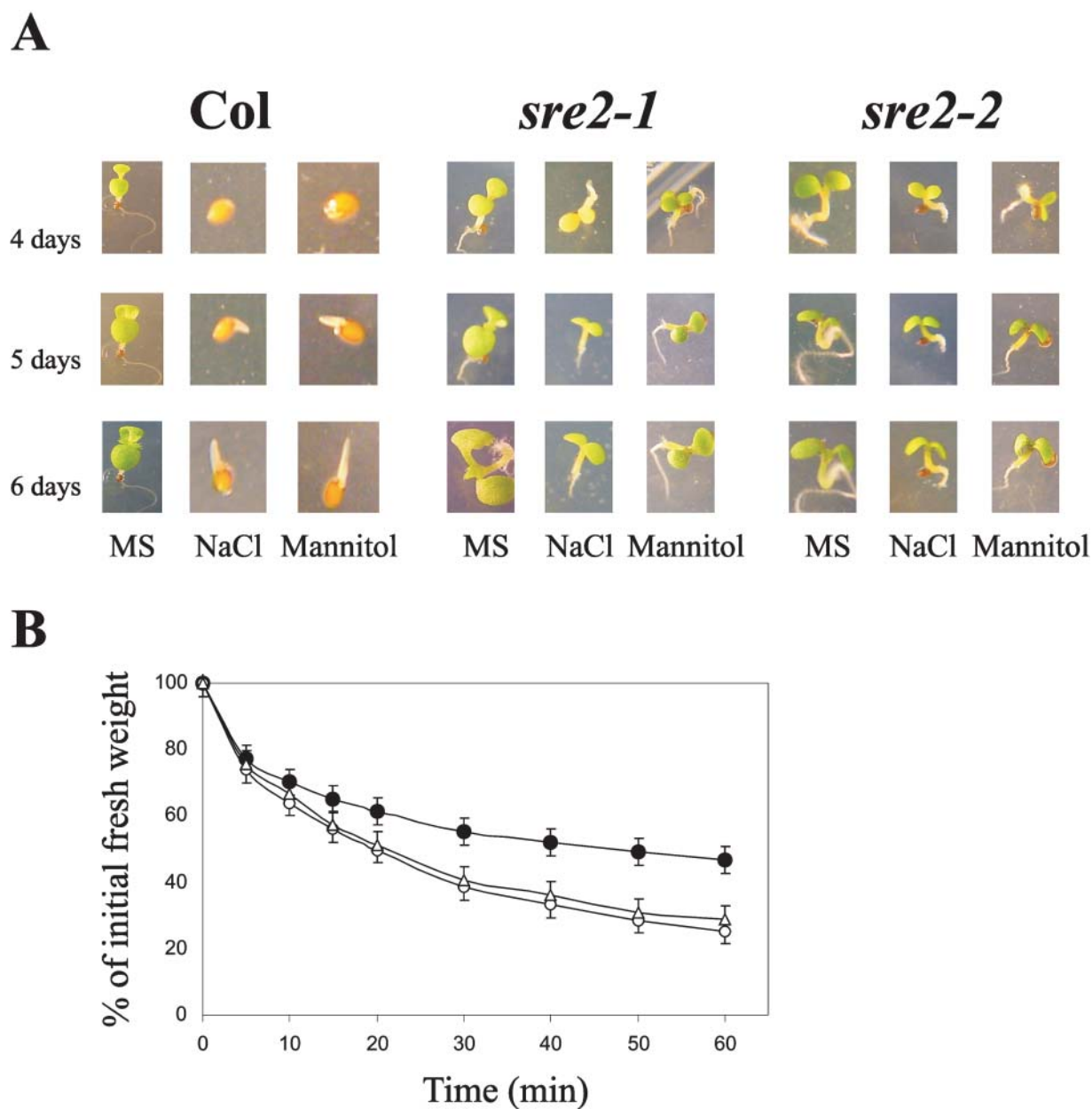
Figure 1. Phenotype of *sre2-1* and *sre2-2* mutants. A, Seed germination in the presence of NaCl and mannitol. Wild-type Col ecotype (left), *sre2-1* (middle), and *sre2-2* (right) seeds were germinated on either MS medium (MS) or medium supplemented with either 200 mM NaCl or 400 mM mannitol. The picture was taken at 4, 5, and 6 d after sowing. B, Enhanced transpiration rate of *sre2-1* (*aao3-2*) and *sre2-2* (*aao3-3*) plants compared to wild type. Percentage of initial fresh weight was measured in detached rosette leaves of either Col (black circles), *sre2-1* (white circles) or *sre2-2* (white triangles) plants.

Sequence analysis of the *AAO3* gene in *aao3-3* revealed a complex mutation that affects a gene stretch from nucleotides 3,066 to 3,080, including deletion of three nucleotides and resulting in several missense mutations (Fig. 2A). This mutation leads to loss of a *Tai*I restriction site, and, consequently, a CAPS marker was developed based on this DNA polymorphism. Analysis of F$_2$ chromosomes of homozygous *aao3-3* individuals revealed cosegregation of the three-nucleotide deletion observed in the *AAO3* gene with the

ABA-deficient phenotype (data not shown). Contrary to the *aao3-2* mutation, the *aao3-3* mutation does not appreciably affect the level of *AAO3* mRNA (Fig. 2B).

The *AAO3* Gene Plays an Important Role in ABA Biosynthesis Both in Leaves and Seeds

The role of AAO3 in ABA biosynthesis had been restricted to leaves on the basis that seed dormancy of

Table I. *Complementation tests of Arabidopsis sre2, aba, and aao3 mutants*

Complementation tests were done by analyzing intercrosses among *sre2*, *aba*, and *aao3* homozygous mutants. The first individual of each cross acted as female. F_1 and F_2 seeds were scored for germination in 150 mM NaCl 5 d after sowing.

Cross (Female × Male)	Generation	Total Seeds Sown	Germinated	χ^2
sre2-1/sre2-1 × *sre2-2/sre2-2*	F_1	154	135	
sre2-1/sre2-1 × Col	F_2	1,377	304	6.26
sre2-2/sre2-2 × Col	F_2	1,444	359	0.015
sre2-1/sre2-1 × *aba1-1/aba1-1*	F_1	107	0	
sre2-1/sre2-1 × *aba2-1/aba2-1*	F_1	112	0	
sre2-1/sre2-1 × *aba3-1/aba3-1*	F_1	113	0	
sre2-1/sre2-1 × *aao3-1/aao3-1*	F_1	89	74	
sre2-2/sre2-2 × *aao3-1/aao3-1*	F_1	85	71	

aao3-1 mutant was unaffected and its ABA levels in mature dry seeds were only reduced by 40% compared to the wild type (Seo et al., 2000a). Additionally, gene expression studies failed to detect the *AAO3* transcript both in siliques and dry seeds from a Col ecotype (Seo et al., 2000b). As both *aao3-2* and *aao3-3* were isolated in a seed germination screening, we reasoned that AAO3 gene product must contribute to ABA biosynthesis in seeds. Indeed, ABA levels in dry seeds or seeds imbibed and treated with 200 mM NaCl of both *aao3-2* and *aao3-3* were approximately one-third of wild type (Table II). We have reexamined the expression level of *AAO3* in siliques using increasing amounts of total RNA. Between 10 and 20 μg of total RNA from siliques was required to detect *AAO3* by northern blot analysis (Fig. 2C). Using only 5 μg, we could not detect the transcript (in agreement with Seo et al., 2000b). Likewise, in dry seeds we were not able to detect *AAO3* mRNA (data not shown); however, upon imbibition and osmotic stress treatment, we could detect it (Fig. 2C).

To further investigate the role of *AAO3* in seeds, we compared the phenotype of all three *aao3* alleles in seed germination assays. The *aao3-1* mutant, as well as *aao3-2* and *aao3-3* mutants, showed an osmotolerant phenotype in seed germination (Table III). However, whereas *aao3-2* and *aao3-3* mutants were able to germinate under 10 μM paclobutrazol, *aao3-1* was not (Table III). Additionally, *aao3-2* and *aao3-3* mutants showed a reduced dormancy, whereas *aao3-1* showed a dormancy similar to wild type (Table III). These data reveal notable phenotypic differences among *aao3-1* and the new *aao3* alleles and suggest that, at least in

a Col genetic background, the AAO3 gene product plays a role in seed ABA biosynthesis.

The demonstration that, for the *aao3-2* and *aao3-3* independent mutant alleles, a heritable change in phenotype is associated with a mutation in *AAO3* suggests that this gene is responsible for the phenotype being studied. Therefore, introduction of a wild-type *AAO3* allele in *aao3-2* and *aao3-3* should complement the phenotype observed in the mutants. The *AAO3* gene driven by its own promoter region (Pro$_{AAO3}$-*AAO3* construct; Seo et al., 2000a) was introduced into *aao3-2* and *aao3-3* mutants by *Agrobacterium tumefaciens*-mediated transformation. Transformants were selected for kanamycin resistance, and a T3 population homozygous for the transgene was obtained. Experiments of seed germination and early seedling growth under high-osmoticum, as well as measures of leaf transpiration, indicated that the phenotype of *aao3-2* and *aao3-3* mutants was complemented upon introduction of a wild-type *AAO3* transgene (Fig. 3). These results confirm that in a Col genetic background, the *AAO3* gene plays an important role in ABA biosynthesis both in leaves and seeds.

DISCUSSION

The availability of ABA-deficient mutants has allowed the substantiation of the role of ABA in different physiological processes. Additionally, these mutants have been very useful to clone the genes that encode the ABA biosynthetic enzymes. Currently, most of these genes have been cloned in Arabidopsis and other plant species, and only the step involving the conversion of all-trans-violaxanthin to 9-cis-violaxanthin or 9-cis-neoxanthin remains to be characterized (Schwartz et al., 2003). The pioneering biochemical studies based on $^{18}O_2$-labeling experiments, together with the characterization of ABA-deficient mutants and subsequent cloning of the corresponding loci, have contributed to our current understanding of ABA biosynthesis.

The last step of ABA biosynthesis is the oxidation of ABA-aldehyde to ABA, which requires the activity of both AAO3 and ABA3 enzymes (Seo et al., 2000a; Xiong et al., 2001). ABA3 is a MoCo sulfurase required for aldehyde oxydase function in ABA biosynthesis (Bittner et al., 2001; Xiong et al., 2001). The sulfide form

Table II. *ABA content (ng ABA/g dry weight)*

ABA levels were measured in dry seeds, imbibed seeds treated with 200 mM NaCl for 24 h, and turgid or wilted rosette leaves of wild-type, *aao3-2* and *aao3-3* plants.

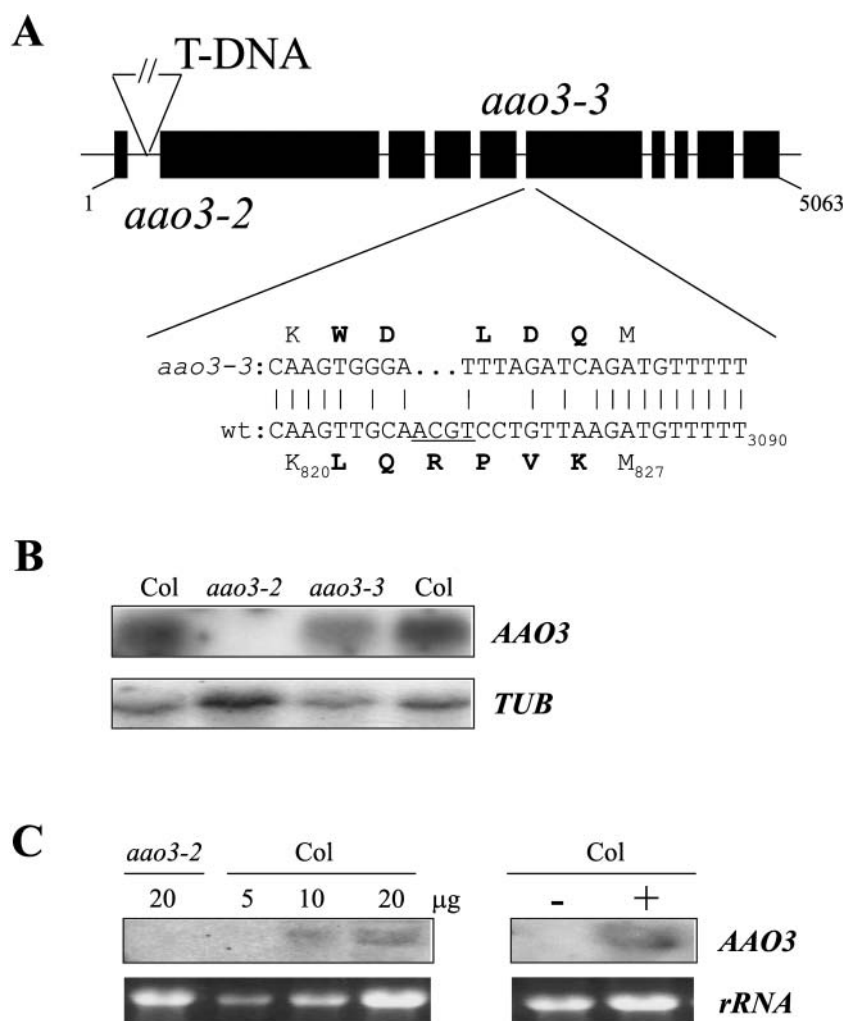|	Dry Seeds	200 mM NaCl	Turgid Leaves	Wilted Leaves
Col	151 ± 15	250 ± 20	103 ± 13	811 ± 76
aao3-2	52 ± 9	83 ± 10	30 ± 3	112 ± 15
aao3-3	57 ± 10	91 ± 12	35 ± 5	162 ± 16

Figure 2. Molecular characterization of the *aao3-2* and *aao3-3* alleles. Northern blot analysis of *AAO3* mRNA. A, Structure of the *AAO3* gene and mutations in the *aao3-2* and *aao3-3* alleles. The position of the T-DNA insertion in *aao3-2* is indicated. The three-nucleotide deletion and missense mutations present in *aao3-3*, as well as the predicted amino acid substitutions, are shown. A *Tai*I restriction site is underlined in the wild-type nucleotide sequence. Amino acid numbering refers to the initial Met residue. Nucleotide numbering refers to the ATG start codon. B, Northern blot analysis of *AAO3* gene expression in Col wild type, *aao3-2* and *aao3-3* mutants. Each track of the blot contained approximately 10 μg total RNA extracted from rosette leaves. RNA analysis was performed as described previously (Gonzalez-Guzman et al., 2002). RNA loading of the gel was quantified by hybridization with a tubulin probe (*TUB*). C, Northern blot analysis of *AAO3* gene expression in siliques (left) and seeds (right). For siliques, increasing amounts (5, 10, and 20 μg) of total RNA from Col wild type and 20 μg total RNA from *aao3-2* mutant were loaded in the blot. For seeds, approximately 20 μg of total RNA were loaded. Total RNA was extracted from seeds that were imbibed for 60 h and then mock-treated (−) or incubated with 400 mM mannitol for 24 h (+). RNA loading of the gel was visualized by ethidium bromide staining.

of MoCo is a cofactor of AAO3, and the AAO3 holoenzyme catalyzes the oxidation of the 1-aldehyde group of abscisic aldehyde to the 1-carboxylic acid group, generating ABA (Seo et al., 2000b). The Arabidopsis aldehyde oxidase family comprises four genes: *AAO1*, *AAO2*, *AAO3*, and *AAO4* (Seo et al., 2000b). Aldehyde oxidase assays indicated that AAO1, AAO2, and AAO4 had almost no activity using abscisic aldehyde as a substrate (Seo et al., 1998, 2000b). Instead, AAO3 oxidized efficiently abscisic aldehyde to ABA (Seo et al., 2000b). Additionally, the isolation and characterization of an ABA-deficient mutant (in a L*er* genetic background) that mapped to the *AAO3* gene provided evidence that this gene is involved in the last step of ABA biosynthesis. Accordingly, *aao3-1* mutant showed a reduced ABA

Table III. *Seed germination and early seedling growth of wild type, aao3-2, aao3-3, and aao3-1 mutants in different media*

Wild-type (Col or L*er* background) and mutant seeds were sowed on MS agar plates supplemented either with 150 mM NaCl, 200 mM NaCl, 400 mM mannitol, or 10 μM paclobutrazol. Approximately 200 seeds were sowed and scored 5 (NaCl) or 8 (mannitol and paclobutrazol) d later. Percentage of seeds that germinated and developed green cotyledons in the different media was scored, and SD values were calculated from three independent experiments. Germination in MS medium was between 95% and 100%. A dormancy assay was performed with freshly harvested seeds (data from last column) by scoring the germination percentage after 5 d in the absence of stratification.

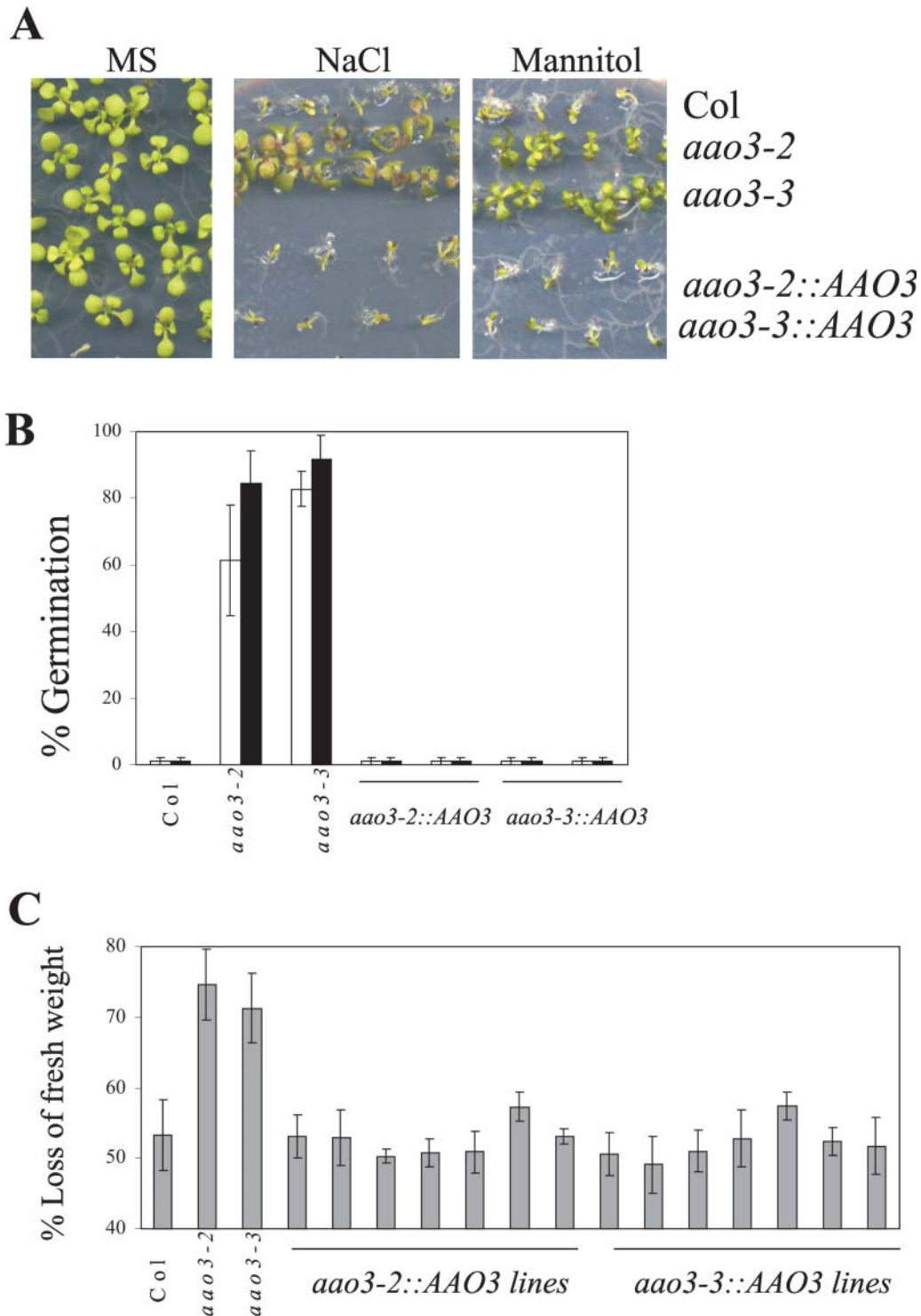|	150 mM NaCl	200 mM NaCl	400 mM Mannitol	10 μM Paclobutrazol	Dormancy
Col	1.0 ± 2.8	0.0 ± 0	0.0 ± 0	0.7 ± 1.2	13.3 ± 3.9
aao3-2	82.3 ± 12.3	61.3 ± 16.5	84.4 ± 10	76.1 ± 19.1	67.9 ± 15.0
aao3-3	88.6 ± 7.7	82.8 ± 5.3	91.7 ± 7.1	86.7 ± 15.1	73.0 ± 18.6
aao3-1	50.7 ± 6.4	28.4 ± 2	91.1 ± 11	3.0 ± 4.2	9.4 ± 3.7
L*er*	6.6 ± 0.7	0 ± 0	0.8 ± 1.1	0 ± 0	5.6 ± 7.5

Figure 3. Complementation of *aao3-2* and *aao3-3* mutants by *AAO3* gene. A, Seed germination and early seedling growth in the presence of NaCl or mannitol. Wild-type (Col), *aao3-2*, *aao3-3*, and T3 homozygous progeny of the mutants transformed with the binary vector pPZP211 containing a Pro$_{AAO3}$-*AAO3* insert. Seeds were germinated on MS medium (MS) or medium supplemented with either 200 mм NaCl or 400 mм mannitol. The picture was taken at 10 d after sowing. B, Percentage of seeds that germinated and developed green cotyledons under osmotic stress for wild-type, *aao3-2*, *aao3-3*, and representative transformed lines (T3 progeny) of *aao3-2* and *aao3-3*. Seeds were sowed on MS agar plates supplemented either with 200 mм NaCl (white bars) or 400 mм mannitol (black bars). Approximately 200 seeds were sowed and scored 5 (NaCl) or 8 (mannitol) d later. Germination in MS medium was between 95% and 100%. C, The enhanced transpiration rate of both *aao3-2* and *aao3-3* plants is complemented by a wild-type *AAO3* transgene. Loss of fresh weight was measured in detached rosette leaves of wild type, *aao3-2*, *aao3-3*, and T1 transformed lines of *aao3-2* and *aao3-3*. Four leaves per individual were excised, and fresh weight was determined at ambient conditions (25°C and approximately 40% relative humidity) after 60 min. The percentage of fresh weight lost is indicated.

level in rosette leaves (20% of wild type in wilted rosettes) and increased transpiration rate compared to wild type. However, *aao3-1* exhibited wild-type dormancy, and ABA levels in dry seeds were reduced only by 40% with respect to the wild type (Seo et al., 2000a). Therefore, the major function of AAO3 was supposed to be the catalysis of the final step of ABA biosynthesis in leaves, but not in seeds (Seo et al., 2000a).

In this work, we report the isolation and characterization of two new alleles of *AAO3*, *aao3-2*, and *aao3-3*, that in contrast to *aao3-1*, showed a reduced dormancy and a significant reduction of ABA levels in dry seeds or seeds submitted to salt stress (Tables II and III). As *aao3-1*, both *aao3-2* and *aao3-3* showed a wilty phenotype and reduced ABA levels in rosette leaves. Molecular characterization of *aao3-2* revealed a T-DNA insertional mutation that abolished mRNA expression of *AAO3*, representing therefore a null allele of *AAO3*. In the case of *aao3-3*, a complex mutation was found that is predicted to result in loss of an amino acid residue as well as several amino acid substitutions ($L_{821}QRPVK_{826}\rightarrow$WDLDQ). The amino acid stretch affected by the mutation lies between the first MoCo binding site (residues 797-803) and a predicted substrate binding site (residues 878-886; Sekimoto et al., 1998). Additionally, the PROSITE program predicts a nucleotide-binding site located between residues 796 and 826, which would be affected by the mutation. Taken together, these data indicate that the *aao3-3* mutation might have a severe effect on AAO3 enzyme activity. Indeed, the phenotypes of *aao3-3* were quite similar to that of *aao3-2*, and ABA content was reduced at similar levels in both mutants.

According to Seo et al. (2000b), *AAO3* gene expression was detected in 8-d-old seedlings, roots, rosette leaves, stems, and flowers; however, *AAO3* mRNA was not detected in dry seeds or siliques. The phenotypes of the *aao3-2* and *aao3-3* mutants prompted us to reanalyze the expression of *AAO3* in siliques and seeds. As a result, *AAO3* transcript was detected in siliques and seeds imbibed and submitted to osmotic stress (Fig. 2C). We had to use at least between 10 and 20 μg total RNA to detect a weak *AAO3* gene expression in siliques. In agreement with Seo et al. (2000b), we could not detect *AAO3* gene expression in dry seeds. However, upon seed imbibition and submission to osmotic stress, expression of the *AAO3* mRNA was induced (Fig. 2C). This result and the increased ABA levels measured in wild-type seeds treated with 200 mM NaCl suggest that ABA biosynthesis takes place in the seed upon osmotic stress. In agreement with that idea, ABA-deficient mutants behave as salt resistant in germination assays (Leon-Kloosterziel et al., 1996; Quesada et al., 2000; Gonzalez-Guzman et al., 2002; this work).

Physiological characterization of both *aao3-2* and *aao3-3* reveals notable differences with respect to *aao3-1*. Although *aao3-1*, *aao3-2*, and *aao3-3* seeds exhibit salt and osmotic stress resistance, only *aao3-2*

and *aao3-3* seeds show a reduced dormancy and paclobutrazol-resistant germination (Table III). These data, together with the reduced ABA levels in seeds of *aao3-2* and *aao3-3*, clearly support a role for the AAO3 gene product in seed ABA biosynthesis. The differential features of *aao3-2* and *aao3-3* with respect to *aao3-1* might be attributed to, at least, two reasons. First, the *aao3-1* mutation might be leaky to some extent. This mutation is a single bp substitution found at the end of the ninth intron of the *AAO3* gene, which results in incorrect splicing of the primary *AAO3* transcript (Seo et al., 2000a). However, shorter transcripts with only a six-nucleotide deletion still occur in *aao3-1* (Seo et al., 2000a). Therefore, a residual activity below the detection limit of the activity staining assay employed cannot be excluded. Second, the *aao3-1* mutation is present in a L*er* background, whereas *aao3-2* and *aao3-3* mutations are in a Col background. The *erecta* mutant has a lesion in a Leu-rich repeat receptor-like kinase that affects development of aerial plant organs (Torii et al., 1996) and also leads to additional unexpected phenotypes (Godiard et al., 2003). Indeed, seeds of L*er* ecotype are more sensitive to inhibition of germination by low exogenous concentrations of ABA than Col ecotype (data not shown). Therefore, the threshold of ABA concentration required to show reduced dormancy could be different in a L*er* than in a Col genetic background. In any case, the identification of genetic lesions in the *AAO3* locus that lead to an ABA-deficient phenotype in seeds of Col genotype strongly suggests that this gene plays a major role in seed ABA biosynthesis.

MATERIALS AND METHODS

Plant Material

Arabidopsis plants (ecotype Columbia) were routinely grown under greenhouse conditions in pots containing a 1:3 perlite-soil mixture. For in vitro culture, seeds were surface-sterilized by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5%) containing 0.05% Triton X-100 for 10 min, and, finally, four washes with sterile distilled water. Stratification of the seeds was conducted during 3 d at 4°C. Afterward, seeds were sowed on Murashige and Skoog (MS) plates (Murashige and Skoog, 1962) containing solid medium composed of MS basal salts and 1% Suc, solidified with 1% agar and pH adjusted to 5.7 with KOH before autoclaving. Different concentrations of NaCl and mannitol were made by adding appropriate amounts of reagents to the basal medium. Plates were sealed and incubated in a controlled environment growth chamber at 22°C under a 16-h light, 8-h dark photoperiod at 80 to 100 μE m^{-2} s^{-1}.

Screening Conditions

T-DNA lines were constructed in the D. Weigel laboratory (Salk Institute, La Jolla, CA) using the pSKI15 vector. Approximately 17,000 lines, stock numbers N21995 and N21991, were provided by the Arabidopsis Biological Resource Center (Ohio State University, Columbus). The distributed T-DNA pools are T4 seeds. Approximately 2×10^5 seeds were screened at high seed density (50 petri plates of 14-cm diameter containing approximately 4,000 seeds per plate) on MS medium (plus 1% Suc) containing 200 mM NaCl. Seeds were considered to be salt-resistant only after they produced fully green expanded cotyledons. Selected salt-resistant candidates (T4) were grown in soil to obtain the T5 progeny for further studies. The T5 progeny of the candidates was retested at low seed density (up to 200 seeds per 9-cm-diameter petri plate) under 150 to 200 mM NaCl.

Genetic Analysis of *sre2* Mutants

Backcrosses of *sre2* mutants to the wild type, intercrosses among *sre2* mutants as well as those of *sre2* with *aba* mutants were performed by transferring pollen to the stigmas of emasculated flowers. F_1 and F_2 seeds were scored for germination in 150 to 200 mM NaCl. In order to map the *sre2-2* (*aao3-3*) locus, homozygous *sre2-2* plants (in a Col background) were crossed to wild-type plants of the L*er* background. From the segregating F_2 generation, homozygous *sre2-2* individuals were selected, and DNA was individually extracted. Mapping of the *sre2-2* locus was carried out by testing linkage with simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994; Lukowitz et al., 2000). The analysis of 44 F_2 chromosomes revealed linkage of the *sre2-2* locus and the nga1126 marker (which is only 150 kb away from *AAO3*).

A CAPS molecular marker (Konieczny and Ausubel, 1993) was developed based on the three-nucleotide deletion present in the *AAO3* gene of *sre2-2*. This mutation destroys a *Tai*I restriction site, and therefore *Tai*I digestion of a 1-kb *AAO3* DNA fragment amplified using the primers F2672 and R3661 (see below) reveals polymorphic bands in *sre2-2* compared to wild type. Thus, *Tai*I digestion of *AAO3* DNA amplified from wild type leads to four DNA fragments (154, 186, 228, and 395 bp). Instead, *Tai*I digestion of *AAO3* DNA amplified from F_2 *sre2-2* individuals leads to three DNA fragments (228, 340, and 395 bp).

Germination Assays

Seeds were plated on solid medium composed of MS basal salts, 1% Suc, and 150 to 200 mM NaCl or 400 mM mannitol. After the indicated days of incubation, the percentage of seeds that had germinated and developed fully green expanded cotyledons was determined. To measure paclobutrazol sensitivity, seeds were plated on medium containing 10 μM paclobutrazol, and germination was determined after 7 d.

ABA Extraction and Determination

Dry seeds, seeds imbibed for 60 h and incubated for 24 h in 200 mM NaCl, as well as rosette leaves of unstressed or drought-stressed plants (unwatered for a week) were ground to a fine powder with mortar and pestle under liquid nitrogen. Duplicate samples (50 mg dry weight each) were extracted with 5 mL 80% acetone containing 100 mg/L 2,6-ditert-butyl-methyl phenol (BHT) and 500 mg/L citric acid, for 16 h at 4°C in the dark. The extracts were further homogenized in a polytron homogenizer at maximum speed for 1 min and centrifuged at 3,000*g* for 5 min. A 1-mL aliquot of the extract was evaporated in a vacuum centrifuge. The sample was resuspended in 100 μL Tris saline buffer (TBS, 50 mM Tris, 1 mM MgCl2, 150 mM NaCl, pH 7.8) and analyzed directly or diluted with TBS in order to fit the ABA content of the extracts within the linear range of the ABA standard curve of the assay. Quantitative analysis of ABA was performed by the indirect ELISA method, using the Phytodetek ABA kit (Agdia, Elkhart, IN).

Molecular Characterization of aao3-2 and aao3-3 Alleles

Plasmid rescue was used to isolate plant DNA flanking sequences of the T-DNA insertion in *aao3-2*. To this end, 5 μg genomic DNA from a *aao3-2* homozygous plant was digested with *Bam*HI (left border rescue). The reaction mixture was extracted once with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1, v/v), once with chloroform:isoamylalcohol (24:1, v/v), and then ethanol precipitated. The DNA was ligated in a 100-μL reaction, and the ligation mixture was precipitated with ethanol. Ligated DNA was introduced by electroporation into the *Escherichia coli* XL-1-Blue MRF' strain (Stratagene, La Jolla, CA). The transformed colonies contained two classes of plasmid. The most common class contained a plasmid whose restriction pattern corresponded to a direct repeat of T-DNA. The second class was represented by the plasmid PR9B. Sequence analysis of the PR9B plasmid revealed that the T-DNA insertion in the *aao3-2* mutant is located at nucleotide 282 of *AAO3*.

In order to identify the mutation occurring in *aao3-3*, oligonucleotides (see below) were designed according to the *AAO3* gene sequence (*At2g27150*), and overlapping fragments encompassing the entire gene were PCR-amplified from *aao3-3*. The amplified products were sequenced on both strands. To avoid errors caused by PCR, three independent PCR samples were mixed and batch sequenced. The following oligonucleotides were used:

F5: 5′AAATCTAACCTTATAATTGG
R1010: 5′ATGTTATGAAGCTCAGCCAC
F900: 5′CAAAGACCATCTTGTAACAT
R1880: 5′ATCAAGACTACATATTCTAT
F1800: 5′CACCTTGCACTCGGAATATA
R2790: 5′GAAGTACTGTGACCCTAGCC
F2672: 5′GCTGAGCGAAAGATAATCTCC
R3661: 5′GGTACTCGAGAAATCCCTCTC
F3550: 5′GATCCTGATGAATATACACTGCC
R4544: 5′CAGTAGTGTACTCTTCCATCATG
F4360: 5′TCCGATATTATTTATGACTGTGG
R5139: 5′TGTAACTTAGCAGCAACGAGAG

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