Aldehyde Oxidase 4 Plays a Critical Role in Delaying Silique Senescence by Catalyzing Aldehyde Detoxification

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The Arabidopsis (Arabidopsis thaliana) aldehyde oxidases are a multigene family of four oxidases (AAO1–AAO4) that oxidize a variety of aldehydes, among them abscisic aldehyde, which is oxidized to the phytohormone abscisic acid. Toxic aldehydes are generated in plants both under normal conditions and in response to stress. The detoxification of such aldehydes by oxidation is attributed to aldehyde dehydrogenases but never to aldehyde oxidases. The feasibility of the detoxification of aldehydes in siliques via oxidation by AAO4 was demonstrated, first, by its ability to efficiently oxidize an array of aromatic and aliphatic aldehydes, including the reactive carbonyl species (RCS) acrolein, hydroxyl-2-nonenal, and malondialdehyde. Next, exogenous application of several aldehydes to siliques in AAO4 knockout (KO) Arabidopsis plants induced severe tissue damage and enhanced malondialdehyde levels and senescence symptoms, but not in wild-type siliques. Furthermore, abiotic stresses such as high and ultraviolet C irradiation caused an increase in endogenous RCS and higher expression levels of senescence marker genes, leading to premature senescence of KO siliques, whereas RCS and senescence marker levels in wild-type siliques were hardly affected. Finally, in naturally senesced KO siliques, higher endogenous RCS levels were associated with enhanced senescence molecular markers, chlorophyll degradation, and earlier seed shattering compared with the wild type. The aldehyde-dependent differential generation of superoxide and hydrogen peroxide by AAO4 and the induction of AAO4 expression by hydrogen peroxide shown here suggest a self-amplification mechanism for detoxifying additional reactive aldehydes produced during stress. Taken together, our results indicate that AAO4 plays a critical role in delaying senescence in siliques by catalyzing aldehyde detoxification.

Aldehyde oxidases (AOs; EC 1.2.3.1) are a multigene family that oxidizes a variety of aldehydes, including abscisic aldehyde and indole-3-acetaldehyde, which can be oxidized to the phytohormones abscisic acid (ABA) and indole acetic acid, respectively (Koshiba et al., 1996; Ori et al., 1997; Seo et al., 1998, 2000a, 2000b; Ando et al., 2006; Zdunek-Zastocka, 2008). The protein architecture of the Arabidopsis aldehyde oxidase (AAO) multigene family is similar to that of the xanthine dehydrogenase family, which is considered to be the ancestor of AAOs (Rodríguez-Trelles et al., 2003) and comprises FAD, Fe-S, and molybdenum cofactor domains as its prosthetic groups (Supplemental Fig. S1; Koshiba et al., 1996). The molecular mass of the A0 monomer is ~145 kD, and the AOs exercise their catalytic function by forming homodimers in animals and homodimers as well as heterodimers in plants (Akaba et al., 1999; Zdunek-Zastocka et al., 2004). In Arabidopsis (Arabidopsis thaliana), four AO genes encode AAO1 to AAO4, and their expression patterns have been shown to be tissue specific: AAO1 is expressed predominantly in seedlings, roots, and seeds, AAO2 in seedlings and roots, AAO3 in rosettes and roots, and AAO4 is abundant in siliques (Seo et al., 2000a; Koivai et al., 2004).

The AOs possess differential substrate specificities that play a key role in identifying and assigning their biological roles (Koshiba et al., 1996; Ori et al., 1997; Akaba et al., 1999; Omarov et al., 1999; Koivai et al., 2000, 2004; Seo et al., 2000a; Ibda et al., 2009). AAO1 and AAO2 homodimers catalyze the oxidation of...
indole-3-acetaldehyde and 1-naphthaldehyde, respectively, with very high efficiency (Akaba et al., 1999), whereas their heterodimer (AAO1::AAO2) exhibits intermediate substrate specificities. The homodimer of AAO3 and its heterodimer with AAO2 oxidize abscisic aldehyde to ABA (Seo et al., 2000b; Koizumi et al., 2004). Among the four AAOs, AAO3 has received special attention owing to its involvement in ABA biosynthesis and its importance during normal and stress conditions (Seo et al., 2000a, 2000b; González-Guzmán et al., 2004). AAO1 was recently shown to be involved in the biosynthesis of indole-3-carboxylic acid (Böttcher et al., 2014). However, the roles of AAOs other than AAO3 still remain largely elusive, especially AAO4.

AAO4 transcript was described as highly expressed in siliques, whereas in rosette leaves, transcript expression was detected only after drought stress (Seo et al., 2000a). While no experiments relating to AAO4 enzyme activity in stressed or unstressed rosette leaves have been described, aldehyde oxidation activity attributed to AAO4 has been shown to be involved in the oxidation of benzaldehyde in siliques, contributing to the synthesis of the benzoic acid pool in this seed capsule (Ibda et al., 2009).

Silicates are sites for seed production and, therefore, require tight control of toxic compounds such as endogenous aldehyde molecules, which are intermediates or by-products of several fundamental metabolic pathways. Aldehydes can be extremely toxic when produced in excess because of their inherent chemical reactivity (Bartels, 2001; Koczeni et al., 2006; Zhang et al., 2012; Biswas and Mano, 2015). These toxic compounds also may lead to lipid peroxidation and reactive oxygen species (ROS) enhancement (Lamb and Dixon, 1997; Bolwell, 1999) to levels that could damage biomolecules such as proteins, lipids, carbohydrates, and DNA, which, in extreme cases, can lead to plant death (Gratão et al., 2005, for review, see Gill and Tuteja, 2010; Mano, 2012).

Under normal physiological conditions, aldehydes are formed constitutively and need to be detoxified (Mano, 2012). In addition, there is an increasing body of evidence for the generation of toxic levels of aldehyde in response to environmental stresses, especially lipid peroxidation-derived reactive carbonyl species (RCS) such as malondialdehyde (MDA), acrolein, and 4-hydroxy-2-nonenal (HNE; Mano, 2012, Biswas and Mano, 2015). These toxic compounds also may lead to lipid peroxidation and reactive oxygen species (ROS) enhancement (Lamb and Dixon, 1997; Bolwell, 1999) to levels that could damage biomolecules such as proteins, lipids, carbohydrates, and DNA, which, in extreme cases, can lead to plant death (Gratão et al., 2005, for review, see Gill and Tuteja, 2010; Mano, 2012).

To elucidate the biological function of AAO4, we first demonstrated that AAO4 is highly expressed in siliques at the transcript and activity levels. Next, we characterized AAO4 activity with various kinds of aldehydes and showed that this enzyme oxidizes an array of aromatic and aliphatic aldehydes and that it differentially generates superoxide (O2−) and hydrogen peroxide (H2O2) in an aldehyde-dependent manner. Importantly, AAO4 in wild-type siliques and AAO4 overexpression in the plant were able to protect plant tissue from toxic levels of aldehydes, including unsaturated RCS such as MDA, HNE, and acrolein, which are products of lipid peroxidation. Siliques of knockout (KO) mutants lacking AAO4 activity, on the other hand, exhibited higher MDA and enhanced chlorophyll degradation levels when exposed to identical levels of the aldehydes. Moreover, KO mutant siliques exhibited premature senescence symptoms, including chlorophyll degradation and 3 to 4 d earlier seed shattering, compared with the wild type. This can be explained by the higher level of RCS such as MDA, HNE, hexanal, and acrolein detected in the mutant’s siliques. Considering also the higher sensitivity of AAO4 KO siliques to dark and UV-C irradiation stresses compared with the wild type, resulting in higher MDA and acrolein levels and increased chlorophyll degradation, it may be concluded that AAO4 plays a major role in protecting siliques against toxic aldehydes. Additionally, AAO4 transcript and activity levels were up-regulated by H2O2 application to siliques, indicating that the ROS generated by the activity of AAO4 may play a role in its self-amplification, enabling further detoxification of toxic levels of aldehydes.

RESULTS

Expression Analysis of AAO4

Northern-blot analyses of AAO4 employing the full-length AAO4 cDNA as a probe have shown that AAO4 mRNA is highly expressed in siliques but detectable in rosette leaves only after drought stress (Seo et al., 2000a). Otherwise, however, only scant data are available regarding AAO4 activity in rosette leaves and siliques (Ibda et al., 2009).

To elucidate the transcript expression and enzyme activity of AAO4 in siliques and rosette leaves, AAO4-overexpressing (OE) plants were generated by introducing a construct containing the complete coding sequence of AAO4 under the control of the cauliflower mosaic virus 35S promoter. The AAO4 coding sequence encoded a polypeptide of 1,337 amino acids with a deduced molecular mass of approximately 147 kD. Two independent transgenic lines with only one insertion of the construct overexpressing AAO4 (OE12 and OE13) were employed for further analyses. KO homozygous
T-DNA insertion plants of AAO4, SALK_057531 (KO31) and SALK_037365 (KO65; Supplemental Fig. S2), were included in the expression analysis as well. AAO4 activity and protein level were examined using in-gel assays and immunoblot analyses. Inclusion of AAO4 KO and AAO4 OE lines in the in-gel analysis in addition to the wild type confirmed the distinct AAO4 activity band, which was additionally corroborated using specific antibodies against AAO4 (see below).

Proteins extracted from 7- to 9-DPA siliques of wild-type, KO31, KO65, OE12, and OE13 plants were fractionated on native PAGE and native/SDS-PAGE as described previously (Yesbergenova et al., 2005). In-gel activity of AAO4 was determined using vanillin, since of all saturated aldehydes, this is the substrate that induces the highest activity, as demonstrated later in this work. No AAO activity was noted after fractionation of the KO siliques protein extracts, whereas only one band of AA0 activity, with a 2- to 4-fold stronger band intensity compared with the wild type, was evident in siliques from OE lines (Fig. 1, A and B, left). An antibody raised against a specific AAO4 synthetic peptide, NAGRHEKLRMGEYLVS (Supplemental Fig. S1, B and C), exhibited pulldown of AAO4 activity in an immunoprecipitation assay with wild-type siliques proteins (Supplemental Fig. S3). The specificity of the antibody was further confirmed when it did not react with any protein in the KO mutant protein extracts in an immunoblot assay but cross reacted with wild-type and OE siliques proteins in a similar pattern (mobility and shape), as was evident in the AAO4 activity bands (Fig. 1, A and B, right).

The presence of AAO4 activity in wild-type rosette leaves was examined with AAO4 OE plants as a positive control. Abundant AAO4 activity was evident in OE rosette leaves, and a faint band with similar mobility was observed in a wild-type extract fractionated by native PAGE; however, none was found when native/SDS-PAGE was employed for the wild-type extract (Fig. 1, C and D, left). Importantly, while the immunoblot analysis confirmed that the activity bands belonged to the overexpressed AAO4, since they exhibited the same band mobility and similar expression level and shape, no immunoblot band was revealed in wild-type rosette leaves by either protein fractionation technique, indicating that the activity band that appeared in the wild-type extract fractionated by native PAGE did not belong to AAO4 but rather to another AAO (Fig. 1, C and D, right).

Quantitative PCR analysis revealed the expression of AAO1, AAO2, and AAO3 transcripts in wild-type, KO31, KO65, OE12, and OE13 siliques and rosette leaves, whereas no expression of AAO4 transcript was evident in KO31 and KO65 mutant siliques and rosette leaves (Fig. 1, E and F). AAO4 transcript was barely detected in rosette leaves of the wild type but was highly elevated in those of OE plants (Fig. 1, E and F). Importantly, while the AAO4 transcript levels were similar in wild-type and OE siliques, the activity and protein levels of AAO4 were more than 3- and 2-fold higher, respectively, in OE than in the wild type. No activity band other than that of AAO4 protein was evident in wild-type and OE siliques detected after native or native/SDS protein fractionation (Fig. 1, A and B). Correspondingly, the relative transcript levels of AAO4 in wild-type and OE siliques were extremely high compared with the other AAO family members, the latter not being affected by the AAO4 mutation in the KO mutants (Fig. 1E).

AAO4 Expression in Drought-Stressed Rosette Leaves

Since no visible AAO4 expression was detected in wild-type rosette leaves whereas northern-blot analyses had previously shown it to be up-regulated after drought stress (Seo et al., 2000a), gradual and drastic drought stress (detached rosette leaves kept for 9 h in a petri dish with or without a transparent cover, respectively) was imposed on rosette leaves to examine AAO4 expression and activity. Quantitative PCR analysis showed that AAO4 transcript was negligible in the unstressed wild-type rosette leaves, while the gradual and drastic drought stresses (28% or less and 47% or less water loss, respectively) did not yield any significant increase in the transcript (Supplemental Fig. S4). However, droplet digital PCR (ddPCR) revealed that, in the wild type, drastic drought imposed on rosette leaves resulted in a 19.5-fold enhancement of AAO4 transcript (from 4.6 to 89.6 molecules of AAO4 per 250 molecules of EF-1α [At5g60390] transcript, as the housekeeping gene product), while in KO plants, only a 1.8-fold increase of AAO4 transcript was detected (from 3.8 to 6.8 molecules per 250 EF-1α molecules; Supplemental Fig. S4C). Despite this significant enhancement in the AAO4 transcript in rosette leaves, no AAO4 protein expression or activity could be detected in wild-type or KO rosette leaves, unlike the activity shown in the unstressed OE rosette leaves (Supplemental Fig. S4D) and wild-type siliques (Fig. 1, A and B). Interestingly, the AAO4 transcript level in the OE plant rosette leaves (108 AAO4/250 EF-1α molecules) that showed AAO4 activity was similar to that in the drought-exposed plant (89.6 AAO4/250 EF-1α) yet was much lower than the level in unstressed wild-type siliques (227 AAO4/250 EF-1α) exhibiting significant AAO4 protein expression and activity (Fig. 1, C and D). These results indicate that, in addition to the transcript expression level, it is necessary to consider the effect of posttranscriptional and posttranslational modification processes on protein expression. Additionally, these results indicate that, if the AAO4 in rosette leaves plays a role in the plant’s response to drought stress, this is manifested through relatively small transcript level changes and/or via modifications below the currently detected protein level.

AAO4 Activity with Various Aldehyde Substrates

Plant AO activity has been widely assessed by employing an in-gel activity assay, and plant AOs have
been shown to oxidize several aldehydes, but with differential ability (Koshiba et al., 1996; Akaba et al., 1998, 1999; Omarov et al., 1999; Koiwai et al., 2000; Seo et al., 2000a, 2000b) that could be indicative of their biological functionality. Using partially purified silique protein with the purification process directed toward benzaldehyde oxidation enhancement, Ibdah et al. (2009) showed that, compared with several other aldehydes, the highest AAO4 activity occurred with benzaldehyde. The enzyme also oxidized several benzaldehyde derivatives as well as indole-3-acetaldehyde but showed no oxidation activity with aliphatic aldehyde, citral, and hexanal (Ibdah et al., 2009).

To gain a better understanding of the function(s) of AAO4, we examined the ability of native AAO4 protein from 7- to 9-DPA developing siliques to oxidize an array of aldehydes. This developmental stage was chosen because the level of constitutively expressed AAO4 was similar to that in 14- or 21-DPA siliques (Supplemental Fig. S5), but unlike the latter, the early-stage siliques did not contain seeds, in which other AAO members may be more strongly expressed (Seo et al., 2000a, 2004). The amount of the fractionated protein extracts and the aldehyde substrate concentration used were verified to be within the ranges that generate a linear response of AAO4 in-gel activity within a certain verified time course (Supplemental Fig. S6). Twenty-five micrograms of total soluble proteins from siliques of wild-type, KO, and OE lines was fractionated by native and native/SDS-PAGE. The two in-gel AO activities after protein fractionation were similar, both showing AAO4 activity with various kinds of aldehydes when detected 20 min from the initiation of the assay (Fig. 2; Supplemental Fig. S7). The reaction medium for AO activity contained MTT and PMS together with 1 mM aldehyde (except for

Figure 1. AAO4 protein and transcript expression in siliques and rosette leaves in wild-type (WT), AAO4 KO (KO31 and KO65), and AAO4 OE (OE12 and OE13) plants. A and B, Twenty-five micrograms of proteins from 7- to 9-DPA siliques was fractionated on SDS (A) and native (B) gels, and AO activity detection (left) and immunoblot analysis employing AAO4-specific antibody (right) were carried out. C and D, One hundred micrograms of protein extract from rosette leaves of wild-type and OE 4-week-old plants was fractionated on SDS (C) and native (D) gels, respectively, and AO activity detection (left) and immunoblot analysis employing AAO4-specific antibody (right) were carried out. Arrows indicate the position of AAO4 protein. E and F, Relative transcript expression of AAO4 as well as other AAOs in siliques (E) and rosette leaves (F). The expression of each AAO in each genotype was compared against AAO1 in the wild type after normalization to the Arabidopsis EF-1α transcript (At5g60390) and is presented as relative expression. Values are means ± se (n = 3). Values denoted with different letters above the bars are significantly different according to the Tukey-Kramer honestly significant difference mean-separation test (JMP 8.0; P < 0.05). Proteins on the SDS gel were reactivated by washing the gel for 1 h in wash buffer containing 10 mM Tris-HCl, pH 7.8, 2 mM EDTA, and 1% Triton X-100. AO enzyme activity was determined in a reaction solution containing 100 mM Tris-HCl (pH 7.5), 1 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 0.1 mM phenazine methosulfate (PMS), and 1 mM vanillin. The reaction was stopped after 20 min for siliques and 2 h for rosette leaves by immersing the gel in 5% acetic acid, and thereafter, gels were scanned and images were captured. KO31 and KO65 are SALK_057531 and SALK_037365, respectively.
HNE and acrolein, where 0.25 and 5 mM, respectively, were employed. Impressively, AAO4 activity in the wild-type protein extract was very high with HNE. In general, activity levels were severalfold higher in OE lines compared with the wild type, and no AAO4 activity was detected in KO lines (Fig. 2; Supplemental Fig. S7). AAO4 exhibited significant in-gel activity with various substrates, such as HNE, vanillin, naphthaldehyde, indole-3-carboxaldehyde, citral, cinnamaldehyde, hexanal, acrolein, benzaldehyde, propionaldehyde, dodecyl aldehyde, salicylaldehyde, and MDA. For further verification of the AAO4 in-gel activity responses to the various substrates, a homologous system for stable overexpression of AAO4 in wild-type rosette leaves, where AAO4 is normally not expressed (Fig. 1, C, D, and F; Supplemental Fig. S4, Figure 2. Aldehyde-dependent activity and ROS production by AAO4 in wild type (WT) and AAO4 modified plants. A, AAO4 in-gel normal and ROS-generated activity in 7- to 9-DPA siliques. The extracted silique proteins were fractionated on SDS gels as described previously (Yesbergenova et al., 2005). AAO4 modified plants included AAO4 KO (KO31 and KO65) and AAO4 OE (OE12 and OE13) plants. B, Similar to A, but the experiment was conducted on different occasions with a different protein lot from the same pool of cryopreserved siliques that was used in A. Normal AAO4 activity was detected in reaction medium containing 1 mM MTT and 0.1 mM PMS in the presence of 1 mM of each of the various aldehydes (left), except acrolein and HNE, where 5 and 0.25 mM were used, respectively. Similar levels of various aldehydes were employed for the detection of ROS-generating activities. \( O_2 \) -generating activity was measured in a reaction medium containing 100 mM Tris-HCl buffer (pH 7.5) and 1 mM MTT (middle), and \( \cdot \text{H}_2\text{O}_2 \)-generating activity was measured in a reaction medium containing 100 mM Tris-HCl buffer (pH 7.5), 2 mg mL\(^{-1}\) 3,3’-diaminobenzidine (DAB), and 4.5 units mL\(^{-1}\) horseradish peroxidase (HRP; right). Arrows indicate the position of AAO4 based on western blot with AAO4-specific antisera as shown in Figure 1. Images of normal AAO4 activity were captured after 20 min, and those of the ROS generation activities of AAO4 were captured 16 h after the beginning of the assay. All gels were scanned, and the intensity of the AAO4 activity band was determined using ImageJ software (http://imagej.nih.gov/ij/). The intensity of the AAO4 activity bands with each aldehyde tested was compared with that obtained with the wild type (employed as a reference; 100%). The zymograms are representative of similar results obtained for at least three independent experiments. Twenty-five micrograms of protein was loaded in each lane to obtain the in-gel AAO4 activity. ND, Not detected.
B–D; Seo et al., 2000a), was employed, and in-gel activity was determined (Supplemental Fig. S8). The Arabidopsis homologous system offers an advantage over heterologous expression in bacteria, as the post-translational modifications are not compromised. Similar in-gel activity responses for the various substrates were obtained for AAO4 overexpressed in rosette leaves and for AAO4 in siliques (compare Supplemental Fig. S8 with Fig. 2).

**O$_2^-$ and H$_2$O$_2$-Generating Activities of AAO4**

The ability of AAOs to generate H$_2$O$_2$ and O$_2^-$ having been demonstrated for AAO1 and AAO3 (Zarepour et al., 2012), we wished to examine whether AAO4 was capable of the same. Additionally, the suitability of various kinds of aldehydes for the generation of ROS by AAO4 was examined, employing aliphatic and aromatic aldehydes after protein fractionation using in-gel assays for O$_2^-$ and H$_2$O$_2$-dependent activity band generation ( Saği and Fluhr, 2001; Yesbergenova et al., 2005). AAO4 was shown to produce O$_2^-$ and H$_2$O$_2$ with different aldehydes (Fig. 2). Among the aldehydes employed, HNE, vanillin, naphthaldehyde, and indole-3-carboxaldehyde appeared to be very efficient in generating O$_2^-$ and H$_2$O$_2$ when siliques were fractionated in both native SDS and native PAGE (Fig. 2; Supplemental Fig. S7). MDA and cinnamaldehyde were relatively low O$_2^-$ producing substrates, especially after native SDS-PAGE, whereas propionaldehyde, acrolein, citral, hexanal, benzaldehyde, and dodecyl aldehyde produced much less O$_2^-$ (Fig. 2A). Interestingly, except for acrolein and propionaldehyde, where the O$_2^-$ generation activity of AAO4 was more prominent than its H$_2$O$_2$ generation activity, for all the other aldehydes tested, the H$_2$O$_2$ generation activity of AAO4 was higher than its O$_2^-$ generation activity (Fig. 2A; Supplemental Fig. S9).

**Histochemical Staining Analyses of O$_2^-$ and H$_2$O$_2$ Generation by AAO4 in Siliques and Rosette Leaves**

In order to validate the results obtained in planta for AAO4 ROS generation, siliques and rosette leaf discs of wild-type and AAO4 modified plants were infiltrated with vanillin and indole-3-carboxaldehyde, after which ROS generation activity was detected as we described previously (Brychkova et al., 2008). No differences were noted between the various mock-treated genotypes (siliques infiltrated with a solution without aldehyde) in their formazan-staining intensity in response to nitroblue tetrazolium (NBT) application, used as a means to estimate O$_2^-$ generation (Brychkova et al., 2008). However, in the presence of aldehydes, significantly stronger formazan staining was seen in siliques of wild-type and OE lines compared with KO lines (Fig. 3A). Unlike in siliques, the intensity of staining was stronger in leaf discs of OE than the wild type and the KO mutant, even for the mock treatment, most likely as the result of AAO4 overexpressed activity employing endogenous aldehydes. This difference was enhanced further when the aldehydes were added to the infiltration solution (Fig. 3B). These results are in agreement with the AAO4-mediated activity pattern obtained with in-gel assays and, thus, demonstrate that AAO4 has the ability to contribute to O$_2^-$ production in planta.

The production of H$_2$O$_2$ also was assessed in planta using DAB staining of plant tissue. A significantly higher H$_2$O$_2$ generation rate was found in siliques of OE lines relative to the wild type, while a significantly lower rate was found in KO mutants compared with wild-type siliques (Fig. 4A). A similar staining pattern was observed when rosette leaves of these genotypes were exam- ined for DAB staining by H$_2$O$_2$ generated owing to the oxidation of infiltrated aldehyde by overexpressed AAO4 (Fig. 4B). These results demonstrate that AAO4 has the capacity to contribute not only to O$_2^-$ but also to H$_2$O$_2$ production in planta.

In vitro assays of the kinetics of AAO4 O$_2^-$ and H$_2$O$_2$-generating activities allowed us to further differentiate between AAO4-mediated ROS production and production facilitated by the activity of other AAOs. The observed pronounced difference in aldehyde-dependent ROS production between wild-type and OE siliques was evident in siliques of OE and wild-type plant tissue 4 h after benzaldehyde or hexanal application to the siliques (Fig. 5, A and B). In particular, siliques of KO plants exhibited complete loss of their chlorophyll 24 h after aldehyde application, whereas siliques of wild-type and OE plants still remained green (Fig. 5A).

**AAO4 Protects Siliques from Senescence Induced by Exogenously Applied Aldehydes**

The possibility that AAO4, which is abundantly present in siliques (Figs. 1 and 2), could protect plant tissue against the toxicity of aldehydes was examined in siliques from wild-type, KO, and OE plants infiltrated with 2 mM benzaldehyde or 5 mM citral, hexanal, or naphthaldehyde. A significant (3- to 4-fold) enhancement in transcript levels of senescence-associated genes, CYSTEINE PROTEASE SENESCENCE-ASSOCIATED GENE12 (SAG12) and SENESCENCE RELATED GENE1 (SRGI), was apparent in KO but not in OE and wild-type plant tissue 4 h after benzaldehyde or hexanal application to the siliques (Supplemental Fig. S10). Importantly, significant chlorophyll degradation was evident in siliques of KO lines already 6 h after aldehyde application, whereas the chlorophyll level in wild-type and OE siliques was hardly affected (Fig. 5, A and B). In particular, siliques of KO plants exhibited complete loss of their chlorophyll 24 h after aldehyde application, whereas siliques of wild-type and OE plants still remained green (Fig. 5A).
Furthermore, levels of the toxic MDA were measured 6 h after aldehyde application as an indicator of lipid peroxidation (Mittler, 2002; Apel and Hirt, 2004) resulting from the toxicity of the aldehydes tested. An ~3-fold enhancement in MDA in response to benzaldehyde treatment and a more than 2-fold increase in treatments with hexanal, citral, or naphthaldehyde were observed in siliques of KO mutants relative to mock-treated KO, OE, and wild-type siliques (Fig. 5C). By contrast, no differences were observed between aldehyde-treated wild-type and OE siliques and the corresponding mock-treated siliques (Fig. 5C). Siliques of wild-type and KO plants were further subjected to the toxic lipid peroxide-derived carbonyls (Matsui et al., 2009; Biswas and Mano, 2015) HNE (0.5 mM) and acrolein (1 mM). KO siliques showed more than 50% loss in chlorophyll and approximately 2-fold increase in MDA, whereas no significant change was noticed in chlorophyll or MDA levels in wild-type tissue after 6 h of acrolein and HNE application relative to the mock (water)-treated siliques (Fig. 6, B and C). Correspondingly, very high expression of the senescence marker genes SAG12, SRG1, and NAC-LIKE, ACTIVATED BY AP3/PI (NAP) was evident in KO siliques, whereas wild-type siliques did not show significant changes in SAG12 and SRG1 and only a moderate increase in NAP in response to exposure to HNE and acrolein for 4 h (Fig. 6D). These results indicate that AAO4 activity protected siliques tissue either by oxidizing the various infiltrated aldehydes and/or by oxidizing toxic carbonyls, such as MDA generated as a result of the toxicity of the infiltrated aldehyde.

Figure 3. AAO4-mediated O$_2^\cdot$ production in siliques and rosette leaves of wild-type (WT) and AAO4 modified plants. A and B, O$_2^\cdot$ generation in 7- to 9-DPA siliques (A) and in 7-mm-diameter rosette leaf discs (B). Discs were carved from 4-week-old wild-type, AAO4 OE (OE12 and OE13), and AAO4 KO (KO31 and KO65) plants. Siliques and leaf discs were infiltrated with either 1 mM indole-3-carboxaldehyde (ICHO) or vanillin and then kept in light for 2 h, followed by infiltration with 50 mM Tris-HCl (pH 7.5) solution containing 0.8 mM NBT for 1 h in the dark. Subsequently, chlorophyll was removed by boiling for 15 min at 80°C in 96% ethanol. Infiltration in solution without aldehyde served as a control (Mock). The results presented are representative of at least three independent experiments. The images were turned to grayscale, and the intensity of NBT staining was quantified using ImageJ software (http://rsbweb.nih.gov/ij). The grayscale ranged from 0 (white) to 255 (complete black). Values are averages ± SE (n = 3). The quantified total relative O$_2^\cdot$ production, presented in bar diagrams as relative intensity with wild-type mock as a reference (100%), is shown below the respective NBT-stained tissue. C and D, Kinetics of O$_2^\cdot$ production during the AAO4-catalyzed oxidation of aldehydes in wild-type, KO65, and OE12 siliques (C) and rosette leaves (D). The kinetic reactions were conducted at 25°C in a total volume of 200 μL consisting of 50 mM phosphate buffer (pH 7.5), 1 mM vanillin, 1 mM epinephrine, and 35 mg mL$^{-1}$ final concentration of protein. Enzyme kinetics was determined by monitoring A$_{480}$ for up to 30 min immediately after adding the aldehyde. The rate of reaction determined in the presence of 24 units of copper/zinc-superoxide dismutase was subtracted from the value in the absence of copper/zinc-superoxide dismutase and is presented in arbitrary units min$^{-1}$ mg$^{-1}$ protein. Results are means of three biological replicates. Values denoted with different letters above the bars are significantly different according to the Tukey-Kramer honestly significant difference mean-separation test (JMP 8.0; P < 0.001). KO31 and KO65 are SALK_057531 and SALK_037365, respectively.
AAO4 Expression in Siliques Exposed to Toxic Levels of Aldehydes

The activity and expression of AAO4 were tracked in siliques from both wild-type and AAO4 modified plants after subjecting to aldehyde treatment. AAO4 activity and expression levels were significantly higher in the OE siliques than in the wild type, while neither transcript nor the activity of AAO4 was detectable in the KO siliques. Importantly, while no significant difference in AAO4 activity was detected between the various mock- and aldehyde-treated wild-type siliques (Fig. 5D, top), protein expression was distinctly higher in the aldehyde-treated siliques (Fig. 5D, bottom). Except for a faint activity band below the major activity band revealed by immunoblot analyses as belonging to AAO4, no other significant AAO activity was visible, confirming that AAO4 is a major AO in wild-type and OE siliques (Fig. 5D).

To gain insight into how the transcriptional regulation of AAO4 and other AAOs takes place in response to the aldehydes tested, expression analysis was carried out in siliques. Transcript levels of AAO4 in mock- and aldehyde-treated wild-type and OE plants remained more than 100-fold higher than for AAO1 and AAO2 and 15- to 20-fold higher than for AAO3 (Supplemental Fig. S11). No transcript of AAO4 could be detected in either mock- or aldehyde-treated KO siliques (i.e. in tissues where no AAO4 was expressed) relative to their respective expression levels in wild-type or OE plants (Supplemental Fig. S11). Aldehyde treatments had a marginal effect, if at all, on AAO1 to AAO3 transcript expression in all the genotypes, and their expression level remained considerably lower than that of AAO4 in siliques exposed to benzaldehyde, hexanal, citral, or naphthaldehyde (Supplemental Fig. S11). On the other hand, the levels of AAO1 and AAO3 (but not AAO2) transcripts increased in response to acrolein or HNE treatment, although AAO4 transcript remained...
100- to 1,000-fold higher (Fig. 6E). Interestingly, in KO siliques lacking active AAO4, AAO1 and AAO3 expression was up-regulated (Fig. 6E); nonetheless, no bands other than AAO4 were detected in the AAO in-gel activity assay or western-blot analyses in siliques exposed to HNE or acrolein (Fig. 6F). Like the AAO4 transcript, the AAO4 protein was up-regulated in response to exposure to the carbonyl aldehydes, reflecting the role of AAO4 in detoxifying these aldehydes in the siliques (Fig. 6F).

**Abiotic Stress Induces Earlier Senescence in AAO4 KO Siliques**

Abiotic stress has been shown to enhance the level of toxic aldehydes in plants (Biswas and Mano, 2015). Here, AAO4 KO siliques exhibited rapid senescence symptoms (enhanced senescence marker transcripts and chlorophyll degradation) in response to exogenous aldehyde application (Figs. 5 and 6). Importantly, UV-C irradiation has been shown to induce an increase in senescence marker transcripts in ripening fruits (Yin et al., 2016), while extended dark stress has been shown to do the same in plant leaves (Brychkova et al., 2008).

To examine the utility of AAO4 for siliques under abiotic stress, wild-type and AAO4 KO siliques were subjected to dark stress and UV-C irradiation (see "Materials and Methods"). Dark stress for 3 d or exposure to 150 mJ of UV-C irradiation resulted in greater loss in chlorophyll and enhanced accumulation of MDA and acrolein in KO than in wild-type siliques (Figs. 7, A–C, and 8, A–C). In both sets of treatments, enhanced transcript accumulation of the senescence marker genes SAG12 (~4-fold in the dark and 500-fold...
or greater in UV-C) and SRG1 (~4- and ~10-fold in the dark and UV-C, respectively) was observed in KO siliques relative to stressed wild-type siliques (Figs. 7D and 8D). NAP transcript remained at similar levels in wild-type and KO siliques after the dark treatment, but in UV-C-induced senescence, its expression was ~3-fold higher in KO mutants than in the wild type. Interestingly, extended dark stress led to significant enhancement of AAO3 transcript in both the wild type and KO, although more so in KO, whereas the transcript level of AAO1, although significantly enhanced in KO, was much lower than that of AAO3. Conversely, although UV-C stress led to some enhancement of AAO3 transcript expression in KO, AAO1 increased more than 25-fold (Figs. 7E and 8E). An important finding was that, despite the already high levels of AAO4 in control wild-type siliques, significant enhancement of its expression was observed under dark and UV-C stress (Figs. 7E and 8E). In-gel activity determination and immunoblotting were performed as described in Figure 1.

AAO4 Activity Protects Siliques against Senescence Induced by Endogenous Toxic Aldehydes

When wild-type and AAO4 mutant plants were examined for phenotypic differences, insignificant differences were found between wild-type, AAO4 KO, and OE plants in terms of plant and silique

Figure 6. Effects of the application of reactive aldehydes on chlorophyll level, MDA content, expression of senescence-related transcripts, and AAO4 expression in siliques of Arabidopsis wild-type (WT) and AAO4 KO plants. A and B, Effects on the appearance of siliques (A) and on relative chlorophyll content (B). Seven- to 9-DPA siliques were infiltrated with 1 mM acrolein and 0.25 mM HNE for 24 h. Water-infiltrated siliques served as a control (Mock). Treated siliques were photographed 24 h after aldehyde application; the photograph presented is representative of at least three independent experiments. Remaining chlorophyll was measured 6 h after aldehyde application. Chlorophyll from untreated mock samples was taken as a reference for individual genotypes. Error bars represent se for two sets of experiments (n = 16). C, MDA content in aldehyde-treated siliques. MDA was determined 6 h after the application of aldehydes. Results are presented as means ± se (n = 3) and represent three different experiments with similar results expressed in nmol g⁻¹ fresh weight (FW). D, Relative transcript expression of the following senescence markers: SAG12 (At5g45890), SRG1 (At1g17020), and NAP (At1g69490). The expression of SAG12, SRG1, and NAP in aldehyde-treated siliques was compared with that in wild-type controls after normalization to the Arabidopsis EF-1a gene product (At5g60390) and is presented as relative expression. E, Relative transcript expression of AAOs. The expression of each AAO was calculated relative to AAO1 in the wild-type control after normalization to the Arabidopsis EF-1a gene product. Values in D and E are means ± se (n = 3). Values denoted with different lowercase letters above the bars are significantly different according to the Tukey-Kramer honestly significant difference mean-separation test (JMP 8.0; P < 0.05). F, AAO4 activity and AAO4 protein level in siliques in response to aldehyde treatment. In-gel activity determination and immunoblotting were performed as described in Figure 1.
morphology, plant biomass, bolting age, seed number per siliqua, average seed weight, and percentage of seed germination (Supplemental Table S1). However, senescence symptoms occurred earlier in KO siliques than in the wild type, with average start of seed shattering at ~20 DPA for KO versus ~24 DPA for wild-type siliques (Supplemental Table S1). For our experiments, six age-matched wild-type and KO siliques were selected to ensure consistency in developmental stages (Fig. 9A). To avoid background activity associated with seeds, developing seeds were removed from each siliqua and the seedless siliques were used for the determinations. The first six KO siliques sampled at 19 DPA exhibited more than 50% chlorophyll loss, versus a marginal loss of 5% in wild-type siliques (Fig. 9, A and B). MDA level in the seedless KO siliqua sampled at 19 DPA was ~40% higher than in six wild-type and mutant seedless siliques sampled a few days earlier (at or before 15 DPA); only an insignificant change in MDA level was detected in wild-type siliques sampled at 19 DPA (Fig. 9C). The morphological senescence symptoms were paralleled by high expression of the senescence marker genes SAG12, SRG1, and NAP. A huge jump in transcript of SAG12 compared with siliques at or before 10 DPA (more than 1,000-fold) and very high enhancement in SRG1 and NAP (~100-fold) were observed in 19-DPA seedless KO siliques (Fig. 9D). By contrast, although transcripts of these genes also increased in wild-type siliques of the same age, the increment was far less than in the KO mutants (Fig. 9D).

We also examined the transcript and protein expression of the AAOs. Although the transcripts of AAO1 and AAO3 in 19-DPA KO seedless siliques were significantly higher than in the control siliques at or before 10 DPA or 19-DPA wild-type seedless siliques, no activity band other than the AAO4 activity band was detected. Interestingly, despite the already high constitutive expression of AAO4, increases in AAO4 transcript as well as in protein level and activity were observed in 19-DPA wild-type seedless siliques (Fig. 9, E and F), indicating the importance of continued elevated expression of AAO4 for delaying siliqua senescence and seed shattering.

As aldehyde detoxification by AAOs had not been shown previously in planta, we were intrigued to find
that siliques of AAO4-impaired plants senesced faster than wild-type siliques and also exhibited elevated MDA levels. Moreover, exposure to toxic levels of aldehyde induced senescence phenotypes in KO but not in wild-type siliques (Figs. 5 and 6). A reasonable conjecture was that the early senescence symptoms were the result of the enhancement of other reactive aldehydes (Biswas and Mano, 2015) that failed to be detoxified by oxidation owing to AAO4 impairment. Indeed, examination of the aldehydes in 15-DPA seedless KO siliques (before yellowing had become discernible) revealed that, while levels of benzaldehyde were not significantly different from the wild-type readings, those of the reactive aldehydes acrolein, HNE, propionaldehyde, and hexanal were significantly higher (Fig. 10). The enhanced levels of the aldehydes, and especially of RCS, in the KO seedless siliques strongly suggest that AAO4 plays a key role in aldehyde homeostasis in siliques.

In an earlier study, self-amplification of tomato (Solanum lycopersicum) AOs by one of its products, ABA, was shown to be feasible (Yesbergenova et al., 2005). Here, after showing that AAO4 produces H2O2 during aldehyde oxidation (Figs. 2 and 4), we proceeded to examine the effect of the resulting product, H2O2, on the regulation of AAO4 expression and activity. We found that the transcript level of AAO4 was enhanced significantly in wild-type siliques after incubation with H2O2 (Fig. 11A); we also found a 2-fold enhancement in AAO4 activity in H2O2-treated siliques compared with mock-treated siliques (Fig. 11B). In view of our finding that AAO4 protects siliques against toxic aldehydes while the absence of AAO4 activity leaves them vulnerable to aldehyde toxicity, these results suggest a possible feedback mechanism whereby the self-amplification of AAO4 induced by the ROS generated by AAO4 enables ongoing protection of the siliques against toxic aldehydes.

**DISCUSSION**

AAOs are known to differentially oxidize a variety of aldehydes, including phytohormone precursors (Seo et al., 1998, 2000a; Akaba et al., 1999; Koizumi et al., 2000, 2004). In view of the broad substrate specificity of this group of enzymes, it is likely that the AAOs have...
additional roles other than in phytohormone biosynthesis. Above a certain threshold level, aldehydes become toxic to cells, since these are highly reactive compounds that covalently bind to proteins and/or DNA; thus, their oxidation by AOs to the corresponding carboxylic acids to reduce toxicity is a distinct possibility (Knox, 1946; Kundu et al., 2007; Negre-Salvayre et al., 2008; Weigert et al., 2008). We have shown here that AAO4 efficiently catalyzes the oxidation of a range of aldehydes, including reactive ones. The KO siliques showed early senescence during normal growth conditions and upon exposure to abiotic stresses such as dark and UV-C, exhibiting higher reactive aldehyde content compared with wild-type siliques. Furthermore, direct aldehyde application caused severe damage to KO siliques while wild-type siliques remained largely unaffected. These findings are discussed in the following sections.

The Response of AAO4 Siliques to Various Kinds of Aldehydes

Examination of the activity of AAO4 using various aldehydes as substrates was carried out by in-gel activity assay, as done before by others for the characterization of various AOs (Koshiba et al., 1996; Akaba et al., 1998, 1999; Omarov et al., 1999; Koiwai et al., 2000; Seo et al., 2000a, 2000b). Among them, Koiwai et al. (2000) and Seo et al. (2000a) demonstrated an identical substrate specificity pattern of AAO (AAO1–AAO3) in-gel activity with crude protein extract compared with recombinant AAOs. Yet here, affinities were
The transcript expression of AAO4 water (control). The transcript expression of AAO4 was treated, respectively. Three independent experiments. C and T denote control and aldehyde substrate. The zymogram is representative of similar results obtained for protein extract from siliques of wild-type plants treated with H2O2. The transcript expression (A) and activity (B) are shown in response to H2O2. The transcript expression of AAO4 in siliques treated with H2O2 was compared with AAO4 transcript expression in water-treated siliques after normalization to the Arabidopsis gene product (At5g60390). Values are means ± se (n = 3). Values denoted with different letters are significantly different according to the Tukey-Kramer honestly significant difference mean-separation test (JMP 8.0; P < 0.05). The in-gel activity assay of AAO4 was carried with NAD+ as a cosubstrate yield a K_m value of 0.5 mM (Supplemental Fig. S6B). The in-gel activity assay for AAO4 revealed that AAO4 is capable of oxidizing several aldehydes, including RCS, such as acrolein, benzaldehyde, HNE, 1-naphthaldehyde, MDA, vanillin, and others (Fig. 2; Supplemental Figs. S7 and S8). We also showed that AAO4 is capable of oxidizing the aliphatic aldehydes citral and hexanal (Fig. 2; Supplemental Fig. S7). These results run counter to earlier data indicating an absence of detectable AAO4 activity on citral and hexanal (Ibdah et al., 2009). Indeed, as shown here, AAO4 mutants have significantly higher hexanal levels in their siliques compared with the wild type (Fig. 10), suggesting the oxidation of hexanal by the active AAO4 in the wild type. Furthermore, we employed an homologous system for constitutive overexpression of AAO4 in wild-type rosette leaves, where normally AAO4 is not expressed (Fig. 1, C, D, and F; Supplemental Fig. S4, B–D). The homologous system offers an advantage over heterologous expression in bacteria, where posttranslational modifications or the need for cofactors may be compromised. In this case, we attained a similar substrate response for AAO4 overexpressed in rosette leaves as for AAO4 in siliques (compare Supplemental Fig. S8 with Fig. 2).

The differences related to AAO4 activity between the results presented here and those reported by Ibdah et al. (2009) may be attributed to their examination of 4- to 6-week-old siliques that included seeds (Ibdah et al., 2009), whereas in our research (i.e. 1-week-old seedless siliques), siliques in which seeds were physically removed were employed. The removal of seeds is critical, as multiple AAOs are known to be present in seeds (e.g. AAO1, which is highly expressed in seeds and would complicate the analysis of AAO4 activity; Seo et al., 2000a, 2004). Additionally, Ibdah et al. (2009), in their AAO4 activity assay, employed NAD^+ as a cosubstrate together with the aldehyde substrate. However, the use of NAD^+ by plants, or for that matter by any eukaryotic AO, is not known (Nishino and Nishino, 1989; Terao et al., 1998; Garattini et al., 2009; Kundu et al., 2012; Zarepour et al., 2012; Li et al., 2014). Moreover, Coelho et al. (2012) recently demonstrated that the lack of residues leading to a flexible loop around the FAD domain in mammalian AO crystal structure can account for the fact that AOs are pure oxidases and cannot use NAD^+ as the final acceptor of reducing equivalents. The addition of NAD^+ as reported (Ibdah et al., 2009) would reveal other activities (e.g. aldehyde dehydrogenases and xanthine dehydrogenase). These enzymes are able to oxidize benzaldehyde and other aldehydes as well (Hesberg et al., 2004; Kotchoni et al., 2006; Urarte et al., 2015) and may have been the source of the recorded NAD-dependent activities. Indeed, our analysis of AAO4 KO showed a complete lack of detectable transcript and protein (Fig. 1, A, B, and E); yet, the results shown by Ibdah et al. (2009) exhibited rates of 55% to

**Figure 10.** Aldehyde contents in wild-type (WT) and AAO4 KO siliques. Siliques at 15 DPA were used to estimate aldehyde levels. The levels of acrolein, benzaldehyde, n-hexanal, HNE, and propionaldehyde are presented. Values denoted with different lowercase letters are significantly different according to the Tukey-Kramer honestly significant difference mean-separation test (JMP 8.0; P < 0.05). FW, Fresh weight.

**Figure 11.** Effects of H2O2 on AAO4 expression in siliques. Wild-type siliques of 10 DPA or less were treated for 30 min with 0.1 mM H2O2 or water (control). AAO4 transcript expression (A) and activity (B) are shown in response to H2O2. The transcript expression of AAO4 in siliques treated with H2O2 was compared with AAO4 transcript expression in water-treated siliques after normalization to the Arabidopsis EF-1α gene product (At5g60390). Values are means ± se (n = 3). Values denoted with different letters are significantly different according to the Tukey-Kramer honestly significant difference mean-separation test (JMP 8.0; P < 0.05). The in-gel activity assay of AAO4 was carried with protein extract from siliques of wild-type plants treated with H2O2 fractionated by native/SDS-PAGE in reaction medium containing 1 mM MTT and 0.1 mM PMS in the presence of 1 mM vanillin as the aldehyde substrate. The zymogram is representative of similar results obtained for three independent experiments. C and T denote control and aldehyde treated, respectively.
70% of benzaldehyde-oxidizing activity compared with the wild type, likely caused by additional activities unrelated to AAO4 but promoted by the presence of NAD+.

In contrast, the AAO4 activity with the various kinds of aldehydes demonstrated here was confirmed by the absence of AAO4 activity bands in the AAO4 mutant and by the manifolds enhancement of AAO4 activity in the overexpression mutants relative to the wild type (Figs. 1 and 2). The reliability of the results was additionally confirmed by the use of highly specific AAO4 antibody that cross reacted only with AAO4 in the wild type and OE but not in the AAO4 mutant (Fig. 1). Indeed, the pulldown of AAO4 by immunoprecipitation assays abrogated its activity in the extract (Supplemental Fig. S3).

AAO4 Generates ROS in a Substrate-Dependent Manner and Is Induced by H2O2

Like the corresponding enzymes in mammals, which efficiently produce both H2O2 and O2−, the Arabidopsis oxidases AAO1 and AA03 have been shown to produce O2− along with H2O2. However, the substrates for ROS generation by these enzymes have not been described in detail (Zarepour et al., 2012, and refs. therein). Employing proteins extracted from wild-type and AAO4 modified Arabidopsis siliques, we found that AAO4 can generate both O2− and H2O2 in an aldehyde-type-dependent manner (Fig. 2; Supplemental Figs. S7 and S9). Moreover, those aromatic aldehydes whose oxidation by AAO4 resulted in the highest normal activity rates (vanillin, naphthaldehyde, and indole-3-carboxaldehyde) also were associated with the highest ROS-generating activity by the enzyme. Importantly, while cinnamaldehyde led to some O2− generation activity only after native PAGE, the other aromatic aldehydes, benzaldehyde and salicylaldehyde, showed negligible ROS generation activities (Fig. 2; Supplemental Fig. S7). Among the aliphatic aldehydes (citral, dodecyl aldehyde, hexanal, and MDA), only MDA exhibited visible O2− generation activity. H2O2 generation, on the other hand, was observed with all these aliphatic aldehydes, reaching a very high level in the case of MDA, surpassed only by the level of H2O2 activity obtained with vanillin, naphthaldehyde, or indole-3-carboxaldehyde (Fig. 2; Supplemental Fig. S7). Except for cinnamaldehyde, the H2O2 generation activity rate of AAO4 was higher than its O2− generation activity for all the aldehydes tested (Fig. 2; Supplemental Fig. S7).

Why does the AAO4 enzyme simultaneously produce both H2O2 and O2−, and why does H2O2 production appear to be more pronounced than the production of O2−? One explanation might be that simultaneous transfer takes place of one or two electrons from the oxidized aldehyde via the FAD domain of the enzyme to molecular oxygen, to generate, preferably, H2O2 in the case of two-electron transfer and O2− in the case of one-electron transfer. Another possibility is that, in AAO4, as in sulfite oxidase (which, like AAO4, belongs to the family of the molybdoenzymes), O2− is generated during sulfite oxidation by the transfer of one electron to molecular oxygen but that H2O2 is detected as a result of the subsequent spontaneous oxidation of O2− (Hänsch et al., 2006; Byrne et al., 2009; Zarepour et al., 2012). In this second scenario, the H2O2 generated by AAO4 would be the result of spontaneous dismutation of the generated O2−.

The function of the ROS generated by AAO4 in the siliques is not yet fully understood but may be at least 3-fold. First, the generation of H2O2 by AAO4 in an aldehyde-dependent manner and the positive regulation of AAO4 transcript expression and activity by H2O2 point to a self-amplification mechanism of AAO4 that serves to detoxify excess reactive aldehydes such as MDA and HNE (Figs. 2 and 11). Second, the generation of the different ROS types by AAO4 and their different levels, which are aldehyde type dependent (Fig. 2; Supplemental Figs. S7 and S9), enable the tight regulation of ROS milieu/redox homeostasis and probably play a signaling role as well (Foyer and Noctor, 2005; Baxter et al., 2014). Third, the H2O2 generated by AAO4 in the siliques also may play a part in directly detoxifying certain aldehydes to be oxidized to their respective carboxylic acids (Kostka and Kwan, 1989; Du and Bramlage, 1993; Sato et al., 2000).

AAO4 Protects Siliques against Toxic Aldehydes

Aldehyde molecules have to be tightly regulated, since, above a certain threshold level, specific for each member of the group, the aldehyde can damage biomolecules such as proteins, lipids, carbohydrates, and DNA, which, in extreme cases, can lead to plant death (Gratao et al., 2005; for review, see Gill and Tuteja, 2010). In such circumstances, a role for the highly expressed AAO4 in siliques in detoxifying excess aldehyde, especially when the expression of other AAOs is barely noticeable, is distinctly possible (Fig. 2; Supplemental Fig. S7). Indeed, when siliques of wild-type and AAO4 modified plants were challenged with toxic levels of various aldehydes (0.5 mM HNE, 1 mM acrolein or MDA, 2 mM benzaldehyde, and 5 mM citral, hexanal, or naphthaldehyde), siliques of KO lines exhibited higher chlorophyll degradation rates compared with wild-type or OE modified plants, while the latter showed few or no symptoms of damage (Figs. 5 and 6; Supplemental Fig. S12, A and B).

The significantly higher MDA contents detected in KO siliques exposed to acrolein, benzaldehyde, citral, hexanal, HNE, or naphthaldehyde compared with AAO4 OE or the wild type constitute additional evidence of the protective role of AAO4 against aldehyde toxicity in these plant tissues (Figs. 5C and 6C). In view of the fact that MDA is a toxic product of lipid
peroxidation, the significantly lower levels of MDA in the wild type and OE are likely to be due to detoxification of the aldehydes through oxidation by AAO4, leading to no or negligible damage in lipids. Alternatively, these low levels could result from direct detoxification by oxidation of both the applied aldehydes and MDA by AAO4, as evident by AAO4’s ability to oxidize these aldehydes (Fig. 2; Supplemental Figs. S7 and S12). This notion is further supported by the significant damage suffered by KO siliques upon exposure to MDA, namely, a 50% greater decrease in the remaining chlorophyll, versus only marginal reduction (less than 5%) in wild-type siliques (Supplemental Fig. S12, A and B).

Interestingly, the expression of AAO4 transcript level and protein expression and activity in siliques, although constitutively already high, rose further on application of the unsaturated reactive aldehydes, but not when saturated aldehydes were applied (compare Fig. 6E with Supplemental Fig. S11 and Fig. 6F with Fig. 5D). This suggests the operation of a mechanism for enhancing detoxification capacity and shielding plant tissue from toxic aldehydes. The increase in the expression of the senescence-related genes NAP, SAG12, and SRG1 in KO mutants in response to reactive aldehyde application points to a coordinated induction of the senescence-related genes in the absence of functional AAO4 (Fig. 6D; Supplemental Fig. S10). The significantly higher sensitivity of KO siliques to the application of aldehydes, and especially acrolein, HNE, and MDA, defined as the most potent lipid peroxide-derived toxic carbonyl compounds (Biswas and Mano, 2015), as well as the induced expression and high activity of AAO4 when RCS are used as substrates, indicate a role for AAO4 in delaying senescence by specifically protecting plant siliques from these aldehydes. At the same time, the significant increase in AAO1 and AA03 transcript expression in KO siliques (compared with the wild type) in response to acrolein and HNE and the absence of detectable AA0 activity other than that of AAO4 (Fig. 6, E and F) may indicate a role for undetectable yet active AA01 and AA03 in signaling and/or in oxidizing toxic aldehydes in siliques. Otherwise, they suggest a futile biosynthesis leading to approximately 1,000-fold transcript enhancement (Fig. 6E) without any benefit for the detoxification of HNE or acrolein.

**AAO4 Delays Silique Senescence in Normally Grown Plants as Well as in Dark- and UV-C-Stressed Siliques: The Case of Endogenous Aldehydes**

There is increasing evidence for the generation of toxic levels of aldehydes in response to environmental stresses, and more especially of toxic levels of lipid peroxidation-derived aldehydes such as MDA, acrolein, and HNE. Stress-induced damage by toxic aldehydes has been described for abiotic stresses such as ozone, chilling, heat, intense light, drought, salinity, and more (Mano et al., 2009, and refs. therein; Biswas and Mano, 2015, and refs. therein). The suppression of aldehyde induced by aldehyde-scavenging enzymes such as aldehyde reductases (Oberschall et al., 2000; Hideg et al., 2003), aldehyde dehydrogenases (Sunkar et al., 2003; Kotchoni et al., 2006), and NADPH:2-alkenal reductase (Mano et al., 2005) has been described. However, protection against aldehyde toxicity by AOs has not been shown previously. Here, we report that exogenously applied toxic levels of aldehydes, including acrolein, HNE, and others, resulted in early senescence symptoms, such as increased chlorophyll degradation, enhanced MDA levels, and significant promotion of the senescence molecular markers SAG12, SRG1, and NAP. As the senescence-associated changes detected in wild-type and AAO4 OE siliques were insignificant, it is concluded that AAO4 can protect siliques against senescence induced by exogenously applied toxic aldehydes.

In the case of endogenously produced toxic aldehydes, only a few sporadic studies have explored the effect of aldehydes on plant tissue in plants modified with aldehyde dehydrogenase, aldehyde reductase, and 2-alkenal reductase (Mano, 2012). Here, we show that AAO4 prevented premature senescence induced by endogenously generated aldehydes in wild-type and KO siliques exposed at 10 DPA to UV-C irradiation and dark stress. In KO siliques, which lacked active AAO4, elevation of the endogenous reactive aldehydes MDA and acrolein resulted in chlorophyll degradation (Figs. 7, A–C, and 8, A–C) as well as enhanced transcript expression of the senescence marker genes SAG12 and SRG1 compared with the stressed wild-type siliques (Figs. 7D and 8D). To this end, the significantly lower acrolein and MDA in wild-type siliques compared with KO siliques indicates that acrolein and MDA are likely among the native substrates of AAO4 in siliques during stresses such as dark and UV-C irradiation.

Importantly, MDA is considered to be the least reactive of the RCS trio comprising HNE and acrolein (Esterbauer et al., 1991; Alméras et al., 2003; Mano, 2012), and it is also a marker for free radical-catalyzed peroxidation and has been used as such for decades (Janero, 1990; Farmer and Mueller, 2013). MDA can exist in several forms: the enolate form at near-neutral pH, which is more stable, and the protonated forms (such as β-hydroxyacrolein), which occur at low pH (such as in the vacuole and apoplast) and are electrophilic and very reactive (Farmer and Mueller, 2013). Analogously to HNE and acrolein, the protonated forms of MDA can form adducts with lipids, free amino acids, proteins, and DNA (Fogelman et al., 1980; Wallberg et al., 2007; Hyvärinen et al., 2014), which can lead to altered cellular responses. In a further indication of the reactivity of MDA, KO siliques infiltrated with 2 mM MDA at 10 DPA exhibited an~50% loss of chlorophyll content within 6 h, whereas in similarly treated wild-type siliques, the chlorophyll was only slightly damaged (~5% loss; Supplemental Fig. S12).
While many aspects of growth and development in Arabidopsis plant organs have been investigated, senescence in siliques has received relatively little attention. Recently, it was reported that delay in silique senescence is associated with ethylene suppression due to the impairment of NAP, a NAC family transcription factor gene (Kou et al., 2012). Early senescence resulting from impairment in the homeostasis of the silique’s reactive aldehyde had not been shown previously. Early senescence symptoms were noticed in siliques of normally grown KO plants exhibiting seed shattering 4 d earlier than wild-type siliques (Supplemental Table S1) and over 50% chlorophyll loss, versus a marginal 5% loss in the wild type (Fig. 9, A and B). These visible senescence symptoms in the KO siliques were corroborated by the greater than 100-fold enhanced expression of the NAP transcription factor, followed by the more than 2,500- and 100-fold enhancement in the expression of the senescence marker transcripts SAG12 and SRG1, respectively, relative to wild-type siliques (Fig. 9D). Detection of reactive and normal aldehydes revealed significantly higher levels of reactive aldehydes in KO siliques compared with the wild type. In addition to MDA, these reactive aldehydes included acrolein and HNE, which were shown to lead to programmed cell death (Biswas and Mano, 2015). The senescence and subsequent enhancement of toxic aldehydes and senescence symptoms/markers in the siliques that were induced by AAO4 impairment provide further evidence that AAO4 plays an important role in delaying silique senescence by aldehyde detoxification. Additionally, they indicate that acrolein, hexanal, HNE, and MDA, but not benzaldehyde, are likely among the native substrates of AAO4 in siliques during senescence. Interestingly, benzaldehyde in siliques, which has been shown to be an important source for benzoic acid in the seeds catalyzed by AAO4 (Ibdah et al., 2009), was at a similar level in both the wild type and AOO4-impaired mutants (Fig. 10), suggesting that additional players such as aldehyde dehydrogenase may participate in the oxidation of benzaldehyde.

CONCLUSION

Several lines of evidence reported here support the notion that AAO4 plays an important role in delaying senescence in siliques by oxidizing toxic aldehydes. First, AAO4 can efficiently oxidize an array of aromatic and aliphatic aldehydes, including the reactive aldehydes HNE, acrolein, and MDA, as well as vanillin, indole-3-carboxaldehyde, 1-naphthaldehyde, citral, hexanal, and others. Second, the exogenous application of benzaldehyde, citral, hexanal, naphthaldehyde, MDA, acrolein, or HNE to wild-type and KO siliques leads to significant tissue damage, enhanced MDA levels, and senescence symptoms in KO but not in wild-type siliques. Third, upon exposing KO and wild-type siliques to UV-C irradiation and dark stress, it was observed that, in the absence of active AAO4, the KO siliques accumulated elevated levels of the endogenous reactive aldehydes MDA and acrolein, inducing a premature senescence phenotype. Finally, it was shown that siliques of normally grown KO plants had significantly higher concentrations of endogenous RCS (HNE, acrolein, and MDA), together with the enhanced senescence phenotype and earlier seed shattering relative to wild-type siliques. Taken together, these results suggest that AAO4 plays a critical role in delaying silique senescence by catalyzing aldehyde detoxification. It was also shown that AAO4 has the ability to generate both O$_2^-$ and H$_2$O$_2$ in an RCS-dependent manner, whereas AAO4 expression is induced by H$_2$O$_2$. The aldehyde species dependence of H$_2$O$_2$ generation by AAO4 may point to a self-amplification mechanism of AAO4 adapted to detoxifying excess reactive aldehyde species.

MATERIALS AND METHODS

Chemicals

Acrolein, benzaldehyde, cinnamaldehyde, citral, DAB, MTT, dodecyl aldehyde, hexanal, HRP, HNE-dimethylacetal, indole-3-carboxaldehyde, MDA, 1-naphthaldehyde, NBT, PMS, propionaldehyde, salicylaldehyde, and vanillin were purchased from Sigma-Aldrich.

Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) Columbia wild type, AAO4 KO, and AAO4 OE lines were used in this study. All the mutants were derived from the Columbia ecotype. Seeds were grown in trays containing low-nutrient soil at 14 h of light/10 h of darkness, 22°C, and 75% to 85% relative humidity under photosynthetically active radiation of 100 μmol m$^{-2}$ s$^{-1}$ as described by Brychkova et al. (2007).

Abiotic Stress Treatments

For dark and UV-C treatments, siliques at 10 DPA or less were detached from wild-type and KO plants and kept in 9-cm petri dishes on wet filter paper. For dark stress, siliques were kept in the dark with intermittent light exposure of 30 min (100 μmol m$^{-2}$ s$^{-1}$) every 24 h for 3 d and then sampled and frozen in liquid nitrogen. For UV-C irradiation, wild-type and KO siliques were exposed to 150 mJ of UV-C irradiation generated by a Cross Linker CL-508 (Cleaver Scientific) instrument and then kept in the normal light/dark regime for 2 to 3 d until the initial appearance of senescence symptoms in KO siliques. For drought stress experiments, mature rosette leaves from 4-week-old plants were detached, weighed, and kept in petri dishes on wet filter paper for 9 h with and without the plate cover for gradual and drastic drought stress, respectively. Thereafter, weight loss was evaluated and leaves were frozen in liquid N$_2$ for further analyses.

Generation and Haplotype Verification of Plants with Modulated AAO4 Expression

Based on AAO4 sequence information (GenBank accession no. AT1g04580), gene-specific primers were designed to isolate the complete cDNA of AAO4, designated as AAO4FLF (5’-AGTGAATTCAGCGGGTCTACTGTTG-3’) and AAO4FLR (5’-TACGGAATTCCTTAAGGATATGTTTCCATCTAAC-3’). Sites for restriction enzymes EcoRI and BamHI, underlined in the primer sequences, were appended at 5’ in AAO4FLF and AAO4FLR, respectively, for cloning. The complete AAO4 open reading frame was cloned in pART7 in the sense orientation to the 35S promoter at EcoRI and BamHI sites. The resulting construct was digested with NotI, and the cassette containing the 35S promoter region and AAO4 was cloned in the binary vector pmLBAR at the NotI site and then introduced into Agrobacterium tumefaciens strain GV3101 as described.
by us previously (Brychkova et al., 2007). Transgenic Arabidopsis plants harboring these constructs were generated by the A. tumefaciens-mediated floral dip method as described by Clough and Bent (1998). Transformed Arabidopsis lines were first selected by resistance to Basta (glufosinate ammonium; Aventis CropScience) and then verified by sequencing the insert that was isolated by PCR using genomic DNA of these transformants as template and pART7R (designed from the pART7 vector sequence – 100 bp downstream from multiple cloning sites) and AAC4 gene-specific primer AAPFPLF (Supplemental Table S2). The copy number of the construct inserted into the genome of Arabidopsis was estimated first by resistance to Basta and spectinomycin (spectinomycin dihydrochloride; Sigma-Aldrich). Finally, quantitative PCR was employed to verify the copy number of the inserted constructs in the genome of the transgenic plants using primers 35S-Fw and 35S-Rw spanning the 35S promoter as done by us previously (Yarmolinsky et al., 2013). Homozygous Arabidopsis modified lines that contained a single-site transgene insertion were used for the experiments.

T-DNA homozygous KO lines for AA04 (A1g04580), SALK_057351 (KO31) and SALK_037365 (KO65), were procured from the Salk Institute. Homozygous lines were selected using PCR screening with the specific primers LBb1.3 and the primers KO31-LP and KO31-RP flanking the insertion site. Similarly, homozygous KO65 lines were identified by PCR using primer LBb1.3 and the primers KO65-LP and KO65-RP. The sequences of all primers are shown in Supplemental Table S2. The PCR products were sequenced to confirm insertion in the desired genes.

**Sequence Analysis**

Sequence analysis was performed with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 310 cycle sequencer (PE Applied Biosystems).

**RNA Isolation, cDNA Preparation, and Real-Time PCR**

To quantify the transcripts using quantitative reverse transcription-PCR, total RNA was prepared using the Aurum Total RNA Mini Kit (Bio-Rad) according to the manufacturer's instructions. The cDNA was prepared in a 10-μL volume containing 350 ng of plant total RNA that was reverse transcribed with an Script cDNA Synthesis Kit using modified MMLV-derived reverse transcriptase (Bio-Rad) and a blend of oligo(dT) and random hexamer primers, according to the manufacturer's instructions. The generated cDNA was diluted 10 times, and the quantitative analysis of transcripts was performed employing the sets of primers shown in Supplemental Table S3 as described previously (Brychkova et al., 2007).

**ddPCR**

ddPCR was carried out using the QX200 ddPCR system (Bio-Rad) according to the manufacturer’s instructions. Briefly, the ddPCR mixture (22 μL) containing 11 μL of a 25× ddPCR Evagreen Master Mix (Bio-Rad), 100 nM of each primer, and 2.5 μL of the 10× diluted template cDNAs as described above. Then, 20 μL of each ddPCR mixture was dispensed into the separate sample wells of the Bio-Rad DG8 disposable droplet generator cartridge, followed by the addition of 70 μL of Droplet Generator oil in each oil well. The cartridge was covered with the DG8 gasket and kept in the Droplet Generator (QX200) for droplet generation. Droplets were transferred to the DG8 cartridge in a 96-well PCR plate. The PCR plate was heat sealed with a Bio-Rad Pierceable Foil Heat Seal at 180°C in the thermocycler (QX1; Bio-Rad). Subsequently, the plate was kept in a thermocycler (Bio-Rad) for PCR with the following cycling conditions: 95°C for 5 min, 40 cycles each consisting of 94°C for 30 s followed by 60°C for 1 min, and one cycle of 98°C for 5 min with a 12°C hold. After PCR, the PCR plate was loaded on the Droplet Reader (QX200; Bio-Rad) for droplet reading. QuantaSoft software (version 1.7.0.917) was used to analyze the ddPCR data.

**Protein Extraction and Fractionation**

Whole protein from Arabidopsis siliques and rosette leaves was extracted as described by Sagi et al. (1998). Concentrations of total soluble protein in the resulting supernatant were determined according to Bradford (1976). Native PAGE was carried out as follows. Samples were subjected to a Mini-Protein III slab cell (Bio-Rad) with a discontinuous buffer system (Laemmli, 1970) on 7.5% (w/v) polyacrylamide separating gels and 4% (w/v) stacking gels. For native/SDS-PAGE, samples were incubated on ice for 30 min in sample buffer with a final concentration of 47 mM Tris-Cl (pH 7.5), 2% (w/v) SDS, 7.5% (v/v) glycerol, 40 mM 1,4-dithiothreitol as the thiol-reducing agent, and 0.002% (w/v) bromophenol blue (Sagi and Fluhr, 2001). The incubated samples were centrifuged at 15,000g for 3 min before loading and subsequently resolved on a 7.5% (w/v) SDS-polyacrylamide separating gel and 4% (w/v) stacking gels. Both SDS-PAGE and native PAGE were carried out using 1.5-mm-thick slabs loaded with 100 μg of leaf or 25 μg of silique proteins unless mentioned otherwise.

**In-Gel AO Activities with Aldehydes and AO-Dependent ROS Production**

Regeneration of the active proteins after denaturing PAGE was carried out by removal of the SDS by shaking the gel for 1 h in 10 mM Tris-Cl buffer (pH 7.8) solution (65 mM of buffer per 1 mL of gel) containing 2 mM EDTA and 1% (w/v) Triton X-100 (Sagi and Fluhr, 2001). AA04 activity was tested for different aldehydes, such as acrolein, benzaldehyde, citral, cinnamaldehyde, dodecyl aldehyde, hexanal, HNE, indole-3-aldehyde, MDA, naphthaldehyde, propionaldehyde, salicylaldehyde, and vanillin, in 50 mM Tris-Cl (pH 7.5) containing 1 mM MTT, 0.1 mM PMS, and aldehydes (concentrations specified where required) as was done previously (Sekimoto et al., 1997, 1998; Akaba et al., 1998, 1999; Omaorov et al., 1999; Kotwal et al., 2000; Seo et al., 2000a). The reaction solution was buffered to pH 7.5, since this was shown to be the optimum pH for testing AA04 activity with vanillin or benzaldehyde as substrate (Supplemental Fig. S13). O2−-generated activities of the fractionated AA04 protein were evaluated on gels by their ability to generate O2•− by the reduction of MTT (Sagi and Fluhr, 2001) following the addition of different aldehydes and 1 mM MTT. The reactions were stopped by immersion of the gels in 5% acetic acid. The quantity of the resulting formazan was directly proportional to enzyme activity during a given incubation time in the presence of excess substrate and tetrazolium salt (Rothe, 1974). H2O2 production was examined by a modified chromogenic HRP assay in which H2O2 serves as the proton-accepting substrate while DAB serves as the proton donor. The modified reaction mixture contained 2 mg mL−1 DAB, 4.5 units mL−1 HRP, and various aldehydes (concentrations specified where required) in 100 mM Tris-Cl (pH 7.5). The reactions were stopped by immersion of the gels in double-distilled water. The gels were scanned, and the intensity of the bands was determined using ImageJ software (http://imagej.nih.gov/ij/).

**The Km for Vanillin as the Substrate for AA04 Activity**

In-gel activity was determined using the wild-type protein and different concentrations of vanillin (0.25, 0.5, 1, 2, 5, 10, and 20 μM). At least three independent in-gel activities were used for each concentration, and an average of the band intensity after 30 min of reaction development was determined. The intensity of bands at various concentrations [S] of vanillin was divided by 30 (min) and considered as V. These values were used to calculate 1/V and 1/S, and a graph was plotted according to Lineweaver and Burk (1934) to determine the Km for vanillin.

**Western-Blot and Immunoprecipitation Analyses of AA04**

Total soluble proteins were resolved by 7.5% native and SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Immune-Blot Membranes; Bio-Rad) as described by Brychkova et al. (2008). These blots were subsequently used for immunoblotting with the specific antibody raised against AA04 synthetic polypeptide, CNAGRHKLRLGELYLS. Primary antibodies were diluted 1,000-fold, and secondary antibodies (anti-goat IgG; Sigma-Aldrich) were diluted 12,000-fold in phosphate-buffered saline. The blots were visualized in Gel-Doc (Bio-Rad) by staining with an ECL detection system, using the SuperSignal West Pico or Femto Chemiluminescent Substrate (Pierce) according to the manufacturer’s instructions. Band intensities were quantified using ImageJ software.

For the immunoprecipitation assay, 30 μg of protein from wild-type silique was incubated with 20 μL (1 mg mL−1) of AA04-specific antibody in Tris-buffered saline for 30 min at room temperature and then kept at 4°C overnight. Fifty microliters of solution containing 30 μL of silique protein extract was added to 10 μL of the immune complex.
supplemented with 20 μL of Tris-buffered saline was used as the control. The 50-μL mixed solutions were then incubated with 50 μL of Immobilized Protein Affinity resin (IPAP 300; Repligen) at 4°C for 2 h with continuous shaking. Following immunoprecipitation, the tubes containing 100 μL of mixed solution were centrifuged at 10,000 × g for 5 min, and the supernatants were used to carry out the in-gel activity analysis.

**Histochemical Staining of Leaf Discs and Siliques for O$_2^-$ and H$_2$O$_2$ Determination**

For leaf disc treatment, discs (7 mm in diameter) were cut out from 4-week-old wild-type and AAO4 modified Arabidopsis plants and then vacuum infiltrated with 0.1 ml solution of vanillin or indole-3-carboxaldehyde. Leaf discs and siliques infiltrated with water only were used as controls (mock). Discs were placed on 90-mm-diameter plates on moistened filter paper under constant light for 2 h. Siliques also were infiltrated with aldehydes, and staining of discs and siliques for the generated ROS was carried out. NBT-based O$_2^-$ and DAB-based H$_2$O$_2$ staining was carried out as shown before (Brychkova et al., 2008). Images were captured with an Epson V750 Perfection Photo Scanner using Adobe Photoshop (http://www.photoshop.com/) imaging software and were quantified using ImageJ software.

**In Vitro Quantification of AAO4-Mediated ROS Production in Siliques and Rosette Leaves of Wild-Type and AAO4 Modified Plants**

Proteins from siliques and rosette leaves of different genotypes were extracted in 50 mM phosphate buffer (pH 7.5) in a 1:5 (w/v) ratio and desalted through Sephadex G-25 columns. Proteins were quantified and used for spectrophotometric determination of ROS. O$_2^-$ quantification was conducted as described by Sagi and Fluhr (2001) with slight modifications. The reactions were conducted at 25°C in a total volume of 200 μL consisting of 50 mM phosphate buffer (pH 7.5), 1 mM aldehyde, 1 mM epinephrine, and 35 ng mL$^{-1}$ final desalted protein. O$_2^-$-generating activity was assessed by monitoring $A_{532}$ for up to 30 min immediately after adding the aldehyde substrate, employing an Epoch Microplate Spectrophotometer (BioTek Instruments).

H$_2$O$_2$ was quantified based on the ability of H$_2$O$_2$ to form red quinoneminium dye (extinction coefficient of 12 ms at 500 nm) visible at wavelength 500 nm by coupling of 3,5-dichloro-2-hydroxobenzene sulfonate and 4-aminoantipyrine in the presence of HRP (Ysesbergova et al., 2005; Brychkova et al., 2015). All the reactions were conducted at 25°C in a total volume of 200 μL consisting of 50 mM phosphate buffer (pH 7.5), 0.85 mM 4-aminooantipyrine, 3.4 mM 3,5-dichloro-2-hydroxobenzene sulfonate, and 35 ng mL$^{-1}$ final concentration of protein in the presence or absence of 1 mM aldehydes and 4.5 units mL$^{-1}$ HRP. H$_2$O$_2$-generating activity was determined by monitoring $A_{500}$ for up to 30 min immediately after adding HRP.

**Aldehyde Toxicity in Siliques**

Aldehyde toxicity was determined by subjecting siliques to various aldehydes. Siliques were vacuum infiltrated with one of the following aldehydes: 1 mM acrolein, 0.25 mM HNE, 2 mM benzaldehyde or MDA, and 5 mM citral, hexanal, or 1-naphthaldehyde. The MDA solution was kept overnight at 25°C before the infiltration. Water-infiltrated siliques served as controls (mock). After the infiltration, the siliques were placed in 90-mm-diameter petri dishes on a filter paper soaked with the respective aldehydes or water (mock) under constant light. Treated siliques were photographed 12 and 24 h after aldehyde or water application. The aldehyde- or water-treated siliques were sampled 6 h after the initiation of treatment for MDA determination and the remaining chlorophyll measurement, using mock-treated siliques as a reference for each individual genotype. AAO4 protein and transcript expression as well as AAO4 activity were determined using siliques sampled 3 h after the application. The data presented are representative of at least three independent experiments with similar results.

**H$_2$O$_2$ Treatment of Siliques and H$_2$O$_2$-Mediated Aldehyde Oxidation**

Siliques from wild-type plants were placed in petri dishes on filter paper presoaked with water (mock) or 0.1 mM H$_2$O$_2$. In addition, thin filter papers presoaked with either water or 0.1 mM H$_2$O$_2$ were placed on the siliques for 30 min, snap frozen in liquid nitrogen, and stored at −80°C for further experiments.

**Chlorophyll Determination**

Total chlorophyll content was measured in extracts of the siliques as described previously for rosette leaves (Brychkova et al., 2008). The remaining content of chlorophyll in siliques was determined as the quantity of chlorophyll per 20 mg in mock (control) samples and was expressed as the remaining chlorophyll (%).

**Determination and Quantification of Aldehydes**

Lipid peroxide-derived carbonyls were determined according to Matsui et al. (2009). Siliques (250 mg) were ground to fine powder in liquid nitrogen and then dissolved in 3 mL of acetonitrile containing 12.5 nmol of 2-ethylhexanal (as an internal standard) and 0.005% (w/v) butylhydroxytoluene. The homogenate was incubated in a screw-capped glass tube at 60°C for 30 min, and the supernatant was collected and filtered through a glass filter. Carbonyl aldehydes in the resulting supernatant were derivatized with 2,4-dinitrophenylhydrazine (DNPH) mix (62.5 μL of acetonitrile containing 20 mM DNPH and 48.4 μL of 99% formic acid) at 25°C for 60 min. Thereafter, 3 mL of 5 mL NaCl and 450 μL of NaHCO$_3$ were added to the mixture, and the DNPH derivatives were passed through a BondElute C18 cartridge (isorbent mass of 200 mg; Varian), which had been preswollen with 2 mL of acetonitrile, to trap pigments. The eluate was vacuum dried and then dissolved in 100 μL of acetonitrile. Fifteen microliters of the redissolved eluate was analyzed using the Thermo Scientific Dionex UltiMate 3000 UHPLC system (http://www.dionex.com/en-us/products/liquid-chromatography/lc-systems/lp-87043.html) with a variable wavelength VWD-3100 detector. The 15-μL aliquot was injected into a Wakosil DNPH-II column (4.6 × 150 mm; Wako Pure Chemical Industries; http://www.wako-chem.co.jp/english) with the following elution conditions at 1 mL min$^{-1}$ flow rate: 0 to 5 min, 100% A (Wako Pure Chemical Industries); 5 to 20 min, a linear gradient from 100% A to 100% B (Wako Pure Chemical Industries); 20 to 25 min, 100% B; and 25 to 45 min, a linear gradient from 100% B to acetonitrile. The detection wavelength was 340 nm, and the column temperature was 35°C. Aldehydes were identified by their retention times. The MDA level in siliques also was measured as described by Havaux et al. (2003) with slight modifications. In brief, 20 mg of siliques was ground in chilled phosphate-buffered saline containing 10% (w/v) TCA and 0.01 mM PMSF. The homogenates were centrifuged (3,000g for 10 min), and the supernatant was incubated with an equal volume of 0.25% (w/v) thio-barbituric acid solution in a water bath (95°C) for 45 min. The tubes containing the mixture were cooled on chilled water and centrifuged at 10,000g for 15 min. Supernatants were taken for spectrophotometric analysis: optical density (OD) was measured at wavelengths 532 and 600 nm for MDA determination, nonspecific absorbance (OD$_{532}$) was subtracted from OD$_{600}$, and the values of the experimental samples were determined against the MDA standard curve.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AT5G20960 (AA01), AT3G45600 (AA02), AT2G27350 (AA03), and AT1G04580 (AA04).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Sequence information of AAO4 showing characteristic domains of AAOs and a unique stretch of sequence used to raise the AAO4-specific antibody.

**Supplemental Figure S2.** Genomic characterization of AAO4 KO mutants.

**Supplemental Figure S3.** Immunoprecipitation of AAO4 activity employing AAO4-specific antibody.

**Supplemental Figure S4.** Effects of drought on the expression of AAO1 to AAO4 in 4-week-old rosette leaves of wild-type and AAO4-KO plants.
Supplemental Figure S5. AA04 activity in wild-type silicates at different developmental stages and seeds collected from 20-DPA silicates.

Supplemental Figure S6. Linearity of AA04 in-gel activity in response to protein level, and \( K_m \) determination of AA04 for vanillin.

Supplemental Figure S7. Aldehyde-dependent activity and ROS production by AA04 in silicates of wild-type and AA04 modified plants.

Supplemental Figure S8. Aldehyde-dependent activity by AA04 in AA04 OE rosette leaves.

Supplemental Figure S9. Visualization after image intensity adjustment of the barely detectable ROS generation activity of AA04 in Figure 2.

Supplemental Figure S10. Relative transcript expression of SAG12 and SGR1 in silicates of wild-type, KO31 and KO65, and OE12 and OE13 plants in response to aldehyde treatment.

Supplemental Figure S11. Relative transcript expression of AA01 to AA04 in wild-type, KO31 and KO65, and OE12 and OE13 siliques treated with water, benzaldehyde, or hexanal.

Supplemental Figure S12. Effects of MDA application on wild-type and KO31 siliques.

Supplemental Figure S13. Determination of the optimum pH for AA04 activity.

Supplemental Table S1. Biomass accumulation, flowering time, seed weight, germination percentage, and silique scattering in wild-type and AA04 modified plants.

Supplemental Table S2. List of primers used for the verification of transgenic plants.

Supplemental Table S3. List of primer sets used to carry out quantitative real-time PCR analysis with Arabidopsis.

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LITERATURE CITED


