Genetic Removal of Tri-unsaturated Fatty Acids Suppresses Developmental and Molecular Phenotypes of an Arabidopsis Tocopherol-deficient Mutant

WHOLE-BODY MAPPING OF MALONDIALDEHYDE POOLS IN A COMPLEX EUKARYOTE

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Malondialdehyde (MDA) is a small, ubiquitous, and potentially toxic aldehyde that is produced in vivo by lipid oxidation and that is able to affect gene expression. Tocopherol deficiency in the vitamin E2 mutant vte2-1 of Arabidopsis thaliana leads to massive lipid oxidation and MDA accumulation shortly after germination. MDA accumulation correlates with a strong visual phenotype (growth reduction, cotyledon bleaching) and aberrant GST1 (glutathione S-transferase 1) expression. We suppressed MDA accumulation in the vte2-1 background by genetically removing tri-unsaturated fatty acids. The resulting quadruple mutant, fad3-2 fad7-2 fad8 vte2-1, did not display the visual phenotype or the aberrant GST1 expression observed in vte2-1. Moreover, cotyledon bleaching in vte2-1 was chemically phenocopied by treatment of wild-type plants with MDA. These data suggest that products of tri-unsaturated fatty acid oxidation underlie the vte2-1 seedling phenotype, including cellular toxicity and gene regulation properties. Generation of the quadruple mutant facilitated the development of an in situ fluorescence assay based on the formation of adducts of MDA with 2-thiobarbituric acid at 37 °C. Specificity was verified by measuring pentafluorophenylhydrazine derivatives of MDA and by liquid chromatography analysis of MDA-2-thiobarbituric acid adducts. Potentially applicable to other organisms, this method allowed the localization of MDA pools throughout the body of Arabidopsis and revealed an undiscovered pool of the compound unlikely to be derived from trienoic fatty acids in the vicinity of the root tip quiescent center.

The in vivo oxidation of polyunsaturated fatty acids and the diverse compounds generated by this process in healthy and diseased organisms are of increasing interest. Higher plants such as Arabidopsis thaliana offer particularly good systems in which to study many aspects of the biology of this type of molecule because of the availability of mutants in fatty acid synthesis and metabolism. Indeed, the complex cellular organization of plants offers many possibilities to explore the physiological effects, genesis, and distribution of nonenzymatic lipid oxidation products at the whole-organism level. The latter aspect, the whole-body distribution of lipid oxidation products at the cell and tissue level, is largely unexplored in complex organisms like plants.

The interest in nonenzymatic products of lipid oxidation stems in part from the fact that they provide markers in pathogenesis (1, 2) and that they may also have powerful biological activities in the control of stress response gene expression (2–5). Additionally, the susceptibility of fatty acids to oxidation is reported to increase in some mutants with decreased thermostolerance (6). In most cases, lipid oxidation has been studied in aerial tissues, where organelles such as chloroplasts and also mitochondria are particularly rich in the trienoic fatty acid α-linolenic acid (7, 8), a fatty acid that is highly susceptible to nonenzymatic oxidation (9).

One of the α-linolenic acid oxidation products that has been studied in some detail in plants in terms of its origin and biological activities is malondialdehyde (MDA)3 (3, 10). MDA is a three-carbon aldehyde that is a major product of the oxidation of many fatty acids with three or more double bonds and is one of the most ubiquitous small molecules produced by lipid oxidation in many organisms (9, 11). In the mature expanded leaves of Arabidopsis, MDA is known to be produced in vivo from at least two genetically separable sources. The principal source, accounting for ~75% of MDA in expanded leaves, is trienoic fatty acids, whereas the origin(s) of the second pool of MDA identified in leaves is currently unknown (10). Like other products of nonenzymatic lipid oxidation in plants, the localization of MDA at the level of tissues and organs is unknown. This latter problem presents a challenge, and a factor limiting the analysis of molecules like MDA in complex multicellular organisms is that it can be difficult to detect individual molecular species without first extracting the compounds and thus destroying tissue structure. Currently, it is not possible to localize the sites of accumulation of discrete molecular products of MDA.

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3 The abbreviations used are: MDA, malondialdehyde; TBA, 2-thiobarbituric acid; HPLC, high performance liquid chromatography.
lipid oxidation in intact plant tissues. We have attempted to solve this problem for MDA.

To localize MDA within the body of plants, several genetic backgrounds with altered abilities to produce MDA would ideally be employed. Fortunately, Arabidopsis mutants exist with either decreased or increased ability to accumulate MDA. For example, the fatty acid desaturase triple mutant fad3-2 fad7-2 fad8 (12) has lower MDA levels in mature leaves compared with the wild type (10). In contrast, the vitamin E2 mutant vte2-1 (13), a loss-of-function mutant in homogentisate phytyltransferase, lacks both tocopherols and redox-active tocopherol pathway intermediates and overaccumulates MDA (14). Although the nonenzymatic oxidation of di-and tri-unsaturated fatty acids is pronounced in the vte2-1 mutant, there is little or no activation of the synthesis of enzymatically derived jasmonates (which are derived from trienoic fatty acids). Furthermore, the transcripts for key jasmonate-responsive marker genes are not up-regulated in vte2-1 relative to the wild type. The vte2-1 mutant strongly overproduces MDA as well as stable phytostanols during early the post-germination phase (14), and the seedlings also show a strong visual phenotype upon germination. Typically, on emerging from the testa, the seedlings display defects in their cotyledons. These defects are asymmetric; they affect one cotyledon more strongly than the other, often leaving this cotyledon poorly developed and with a bleached appearance (13).

We compared the phenotypes and MDA levels in seedlings of the vte2-1 mutant and the fad3-2 fad7-2 fad8 triple mutant (hereafter termed fad3 fad7 fad8) with those of a novel quadruple mutant (fad3-2 fad7-2 fad8 vte2-1, hereafter termed fad3 fad7 fad8 vte2) derived from crossing the two parents. The results showed that the oxidation of trienoic fatty acids is specifically responsible for the vte2-1 phenotype, including cotyledon defects and up-regulation of GST1 expression. Furthermore, liquid chromatography analysis indicated that the only small 2-thiobarbituric acid (TBA)-reactive substance produced more, liquid chromatography analysis indicated that the only small 2-thiobarbituric acid (TBA)-reactive substance produced more, liquid chromatography analysis indicated that the only small 2-thiobarbituric acid (TBA)-reactive substance produced more, liquid chromatography analysis indicated that the only small 2-thiobarbituric acid (TBA)-reactive substance produced more, liquid chromatography analysis indicated that the only small 2-thiobarbituric acid (TBA)-reactive substance produced more, liquid chromatography analysis indicated that the only small 2-thiobarbituric acid (TBA)-reactive substance produced more, liquid chromatography analysis indicated that the only small 2-thiobarbituric acid (TBA)-reactive substance produced more.

The homozygous fad3 fad7 fad8 mutants were isolated from the F₂ progeny (960 plants) by gas chromatography analysis for 18:3 (15). Ten plants homozygous for the fad3 fad7 fad8 mutations were detected. Three vte2 heterozygotes were detected among these plants. Homozygous vte2 mutants were isolated from the following (F₂) generation by cleaved amplified polymorphism sequence analysis. For VTE2/vte2 differentiation, a 632-bp-long fragment was amplified with forward primer 5′-TTTCACCTGGCACTTTGGAGTTAAG-3′ and reverse primer 5′-AAGTGGCAACTGTGGTAGTAGAAG-3′ in a PCR carried out at an annealing temperature of 51 °C. Restriction enzyme digestion of the vte2 allele with SacI endonuclease (Fermentas, Ontario, Canada) generated two fragments (477 and 154 bp), whereas VTE2 was not digested. Three quadruple mutant lines were obtained. Two of these lines (733 and 782) were propagated. Seed production from the quadruple mutants was rescued by spraying with methyl jasmonate (30 μM, sonicated in water containing 0.01% (v/v) Tween 20) at 2-day intervals. At the F₃ generation, sufficient seeds were obtained for experiments.

RNA Gel Blotting—Total RNA was extracted from 2-day-old seedlings grown on wet filter papers at 20 °C during a 12-h photoperiod (100 μmol of photons m⁻² s⁻¹). RNA was extracted as described (5), separated by electrophoresis, and electroblotted onto nitrocellulose membranes (Amersham Biosciences). The membranes were probed with a cDNA corresponding to the GST1 gene At1g02930 (5).

MDA Quantification—Seedlings (2 days old, 200 mg fresh weight) were harvested frozen in liquid nitrogen and ground to a fine powder. MDA levels in different mutants of Arabidopsis were quantified by a gas chromatography/mass spectrometry method as described (10, 16). The internal standard for quantification was D₂-MDA generated from (2D₂)-1,1,3,3-tetraethoxypropane (Dr. Ehrenstorfer GmbH, Augsburg, Germany) as described (10).

Fluorescence Detection of Aldehyde-TBA Adducts—A fluorescence filter with a 515 ± 10-nm excitation filter and a 555 ± 15-nm emission filter was constructed by Leica Microsystems (Glattingbrugg, Switzerland) based on fluorescence values in Ref. 17. Fluorescence was detected with a Leica MZ16 FA microscope and photographed with a Leica DC300F camera.

TBA Treatments—Plant tissues were treated at 37 °C for 5 h with either 35 mM TBA (Sigma) or 35 mM trichloroacetic acid (Merck) as a control. Tissues were examined by microscopy within 1 h of the end of the 5-h staining period.

HPLC Analysis—MDA-TBA adducts were separated based on Res. 18. All solutions and mobile phases were prepared in purified water (NANOpure, Skan AG, Basel–Allschwil, Switzerland). Wild-type (Col-0), vte2-1, and fad3 fad7 fad8 seeds were germinated in the dark in water for 3 days. These seedlings were then incubated in 35 mM TBA solution (0.02 g of seeds (fresh weight)/ml) for 5 h at 37 °C. The seedlings were ground in liquid nitrogen and extracted in 5% (w/v) trichloroacetic acid in the presence of 0.1% (w/v) butylated hydroxytoluene (0.02 g of seeds/ml). The suspensions were shaken for 5 min at room temperature and then centrifuged at 10,000 × g for 2 min. The supernatant was directly analyzed by HPLC. A standard MDA-TBA adduct was prepared from 5 μmol 1,1,3,3-tetraethoxypro-
pane (Sigma) incubated in 35 mM TBA for 5 h at 37 °C. The reaction was diluted in 5% (w/v) trichloroacetic acid and 0.1% (w/v) butylated hydroxytoluene for injection. HPLC was performed using a LaChrom Elite HPLC system (Merck-Hitachi, Dietikon, Switzerland): L-2100 pump, L-2200 autosampler, L-2300 column oven, L-2450 diode array detector, and L-2480 fluorescence detector. HPLC was controlled with the EZChrom Elite Program Version 3.1.7 (Merck-Hitachi). Separation was performed by injection of a 10-μl sample into a ChromCart column (Nucleosil 120-5 C18, 5 μm, 4 × 250 mm; Macherey-Nagel, Ohringen, Switzerland). The autosampler was maintained at 4 °C and the column at 26 °C. The mobile phase was 24% (v/v) acetonitrile (Merck) in 0.1% (v/v) trifluoroacetic acid (Fluka, Buchs, Switzerland) run isocratically for 8 min at a flow rate of 1 ml/min. Detector wavelengths were set to 515 nm (excitation) and 555 nm (emission). The MDA-(TBA)2 adduct eluted at 21.7 min.

The solvent system was then changed to 0–100% (v/v) methanol in 0.1% (v/v) trifluoroacetic acid (same flow rate and fluorescence parameters). The injected volume was 2 μl. The mobile phase was 24% (v/v) acetonitrile (from 24 to 100% (v/v) in 0.1% trifluoroacetic acid (Fluka, Buchs, Switzerland) run isocratically for 8 min at a flow rate of 1 ml/min. The MDA-(TBA)2 adduct eluted at 21.7 min.

**Liquid Chromatography/Mass Spectrometry Analysis—**Ultra-HPLC/time-of-flight mass spectrometry analyses were performed on a Micromass LCT Premier time-of-flight mass spectrometer (Waters, Milford, MA) with an electrospray interface and coupled with an ACQUITY UPLC system (Waters). Electrospray ionization conditions were as follows: capillary voltage, 2500 V; cone voltage, 90 V; microchannel plate detector voltage, 2650 V; source temperature, 120 °C; desolvation temperature, 250 °C; cone gas flow, 10 liters/h; and desolvation gas flow, 550 liters/h. Detection was in the negative ion mode in the m/z range 100–1000, with a scan time of 0.25 s, and interscan delay of 0.01 s, and a centered mode. A solution of leucine/enkephalin (Sigma) was used for the lock mass. Separation was carried out on a Waters ACQUITY UPLC BEH C18 column (50 × 1.0-mm inner diameter, 1.7 μm). Gradient analysis was performed at a flow rate of 0.25 ml/min with solvent system A (0.1% formic acid and water) and solvent system B (0.1% formic acid and acetonitrile) producing a gradient of 5–98% solvent B in 3.0 min. The injected volume was 2 μl.

**Chemical Phenotype of vte2-1—**Wild-type (Col-0) and vte2-1 (13) seeds were imbibed in water at 20 °C during a 16-h photoperiod (100 μmol of photons·m−2·s−1) for 1 day. The seedlings were then incubated on wet filter papers for 2 days in the presence of MDA (0 or 1 μmol) in 1-liter glass jars during the same 16-h photoperiod and at the same temperature. MDA was generated from 1,1,3,3-tetraethoxypropane by addition to freshly prepared 1 M HCl (100 μl) at room temperature. The MDA solution was applied to cotton wicks. For the wild-type control (0 μM MDA), only HCl was applied. vte2-1 seedlings (1 day post-imbibition) were incubated under the same conditions but in the absence of MDA. After 3 days, seedlings were photographed under the Leica MZ16 FA microscope with the Leica DC300F camera using IM50 software (Leica Microsystems).

**RESULTS**

**Genetic Suppression of the vte2-1 Seedling Phenotype—**During germination, the tocopherol-deficient mutant vte2-1 exhibits a strong phenotype that includes a failure to fully expand one (or, more rarely, both) cotyledons. Additionally, the affected cotyledon is often devoid of chlorophyll (13). Moreover, this phenotype is correlated with the accumulation of potentially toxic compounds derived mainly from the nonenzymatic oxidation of trienoic fatty acids (10, 13). To test whether the vte2-1 phenotype is specifically associated with trienoic fatty acids, two fad3 fad7 fad8 vte2 quadruple mutant lines were isolated from a cross between the trienoic fatty acid-deficient mutant fad3 fad7 fad8 vte2-1 (12) and vte2-1 (see “Experimental Procedures”). Three days after germination of the tocopherol-deficient vte2-1 seedlings, the plants were smaller than Col-0 and displayed a clear vte2-1 phenotype (Fig. 1) (13). However, the wild-type/ fad3 fad7 fad8 triple mutant visual phenotype was restored in the two lines of the quadruple mutant (Fig. 1). The growth of seedlings of quadruple mutants (lines 733 and 782) appeared to be highly similar to that of the wild type and the fad3 fad7 fad8 triple mutant, and no bleaching was observed on the cotyledons of the fad3 fad7 fad8 vte2-1 quadruple mutants. Indeed, none of the fad3 fad7 fad8 vte2 quadruple mutant seedlings presented the cotyledon defects that typify the vte2-1 mutant at this stage of growth.

**MDA Levels and GST1 Gene Expression in Different Arabidopsis Mutants—**The vte2-1 phenotype in seedlings is associated with a strong increase in nonenzymatic lipid peroxidation, resulting in the production of hydroperoxy and hydroxyl fatty acids, linear phytosteranes, and MDA (13, 14). Moreover, the accumulation of these compounds in the vte2-1 mutant correlates with the up-regulation of 160 genes (14). To further test the correlation between the accumulation of trienoic fatty acid oxidation products and the modification of gene expression, MDA levels were quantified, and the transcript levels for GST1, an oxidative stress marker gene (19), were monitored in 2-day-old seedlings of the different mutants (Fig. 2).
seedlings, the MDA concentration increased strongly to levels 14-fold higher than in Col-0 and 22-fold higher than in the fad3 fad7 fad8 triple mutant. In the fad3 fad7 fad8 vte2 quadruple (lines 733 and 782) mutants, we observed a lower MDA level than in the wild type. This level was comparable with the MDA level in the fad3 fad7 fad8 triple mutant. The restoration to a nearly wild-type visual phenotype in the quadruple mutant correlated with a low MDA level. To further test the correlation between the accumulation of trienoic fatty acid oxidation products and gene expression observed in the vte2-1 mutant, the expression of the GST1 gene was analyzed.

**Chemical Phenotype of vte2-1**—To further investigate the potential role of α-linolenic acid oxidation products in the vte2-1 phenotype, we attempted to chemically phenocopy the vte2-1 mutation. This was achieved by exposing germinated seeds of Col-0 to MDA. Wild-type seedlings exposed to MDA partially resembled vte2-1 seedlings and displayed one bleached cotyledon and shorter and narrower hypocotyls (Fig. 3C).

**Development of an in Situ Test to Detect MDA in Plant Tissues**—To facilitate the in situ microscopic detection of MDA, a custom fluorescence filter was constructed. The filter parameters were based on specific fluorescence values for the adduct between MDA and TBA (17), with a 515 nm excitation filter and a 555 nm emission filter. The filter was first tested on germinating Col-0 and vte2-1 seedlings to see whether fluorescence could be observed in the seedlings. A stronger fluorescence in vte2-1 than in wild-type seedlings was expected because of the higher measurable levels of MDA in the former. Fig. 4 shows wild-type (Col-0) and vte2-1 seedlings (3 days post-germination) were incubated at 37 °C for 5 h in 35 mM TBA solution. A weak red coloration at the root tips corresponding to TBA adducts was observed under white light in the wild type and in the vte2-1 mutant (upper panels). Fluorescence (fluor.) was detected at 555 nm (lower panels). Scale bars = 1 mm.
days post-germination) that had been incubated with 35 mM TBA for 5 h at 37 °C and then observed by microscopy. Under white light, a weak red coloration was observed in the root tips of both Col-0 and the vte2-1 mutant. In parallel, the fluorescence at 555 nm was observed in each genotype. For wild-type seedlings, strong fluorescence was observed at the root tip, with weaker fluorescence in the cotyledons. On the other hand, in the vte2-1 mutant, the fluorescence was present throughout the entire seedling. This observation is consistent with the fact that the MDA level in the vte2-1 mutant is 14-fold higher than that in Col-0 3 days after germination (14).

At this point, it was not certain that the fluorescence observed emanated specifically from MDA-(TBA)_2 adducts. To test for the specificity of fluorescence in the vte2-1 mutant, extracts of this plant previously incubated with TBA (for 5 h at 37 °C) were analyzed by HPLC. Using the same wavelengths as used for the fluorescence microscopy, the HPLC chromatograms (Fig. 5) revealed a single peak for the synthetic reference adduct. Only one fluorescent peak was detectable by HPLC analysis of wild-type and fad3 fad7 fad8 triple mutant seedlings, and a larger single peak was observed with vte2-1. These peaks migrated at the same retention time as the synthetic MDA-(TBA)_2 adduct. More extensive HPLC analysis of the wild type using gradients of methanol or acetonitrile revealed only one peak corresponding to the MDA-(TBA)_2 adduct from wild-type plants. Liquid chromatography/mass spectrometry was used to verify the identity of the MDA-(TBA)_2 adduct produced in both the wild-type and fad3 fad7 fad8 triple mutant samples. The mass for the adduct obtained in the negative ion mode (M – H = 322.99) corresponded to that for the synthetic adduct (Fig. 6).

In Situ MDA Detection in Different Arabidopsis Mutants—On the basis of HPLC analysis of plants treated with TBA and the finding of a single dominant fluorescent species corresponding to MDA-(TBA)_2 in wild-type and vte2-1 and fad3 fad7 fad8 triple mutant plants, we used these mutants, together with the fad3 fad7 fad8 vte2 quadruple mutant, to test the in situ microscopic assay in a genetic context. To control for the possible effects of pH on intrinsic fluorescence, seedlings were incubated with trichloroacetic acid (35 mM). Trichloroacetic acid does not form fluorescent adducts with MDA. No fluores-
cence was observed upon this treatment (Fig. 7). In parallel, wild-type (Col-0) and vte2-1, fad3 fad7 fad8 triple, and fad3 fad7 fad8 vte2 quadruple mutant seedlings were incubated with TBA (35 mM, 5 h, 37 °C). As expected, weak cotyledon fluorescence was observed in the wild type (Col-0). Similar but weaker cotyledon fluorescence was observed in the fad3 fad7 fad8 triple mutant. Similar levels of fluorescence were detected, however, in the root tips of the wild-type and fad3 fad7 fad8 triple mutant plants. In contrast to the wild type and fad3 fad7 fad8 triple mutant, very strong fluorescence was observed throughout the body of the vte2-1 mutant. This was largely eliminated in the fad3 fad7 fad8 vte2 quadruple mutant, in which residual fluorescence resembled that in the fad3 fad7 fad8 triple mutant (Fig. 7).

DISCUSSION

Trienoic Fatty Acids Underlie the vte2-1 Seedling Phenotype—Massive nonenzymatic oxidation of di- and trienoic fatty acids occurs shortly after germination in the vte2-1 mutant. This contrasts to the levels and activities of jasmonates, which are enzymatically derived from trienoic fatty acids (20). No evidence was found for the activation of jasmonic acid production or signaling in germinating vte2-1 seedlings (14). Supporting this, vte2-1 aos (allene oxide synthase) double mutants retain the vte2-1 phenotype.4

High levels of linoleic acid 9- and 13-hydroxides accumulate in vte2-1 seedlings, and chiral analysis confirmed their nonenzymatic origin. The corresponding products from α-linolenic acid occur at lower levels (13, 14). Further investigation revealed the accumulation of two other markers of nonenzymatic fatty acid oxidation in vte2-1. These were stable trihydroxyphytoprostanes and MDA, a small reactive aldehyde (14). Together, the data show that both linoleic acid (18:2) and α-linolenic acid (18:3) undergo extensive nonenzymatic oxidation in vte2-1 seedlings. However, which of these two fatty acids contributes to the phenotype of the mutant? This question was addressed herein by combining vte2-1 with a genetic background lacking trienoic fatty acids (18:3 and 16:3). The new experiments showed that the visible vte2-1 phenotype was suppressed in the fad3 fad7 fad8 vte2 quadruple mutant seedlings and was close to a fad3 fad7 fad8 triple mutant phenotype. This demonstrated that trienoic fatty acids are responsible for the vte2-1 phenotype.

What causes the severe damage seen in germinating vte2-1 seedlings? The removal of 18:3 alone in fad3 fad7 fad8 vte2 quadruple mutants was sufficient to suppress tissue damage. This correlated with suppression of MDA overaccumulation because quadruple mutant lines 733 and 782 contained only 4 and 4.5%, respectively, of the MDA level measured in the vte2-1 mutant (Fig. 2). It is likely that a variety of reactive compounds derived from trienoic fatty acids (and including MDA) contribute to the visual phenotype seen in vte2-1. Indeed, reactive compounds containing α,β-unsaturated carbonyl groups (reactive electrophile species) are known to reduce chlorophyll fluorescence, and their overproduction in vivo was predicted to damage plant cells (5, 21). This and the fact that treatment of

4 L. Méne-Saffrané, unpublished data.
wild-type seedlings with MDA was sufficient to induce cotyledon bleaching (Fig. 3) strongly support the hypothesis that reactive compounds derived from 18:3 underlie the vte2-1 phenotype.

Apart from a strong visual phenotype, gene expression is also strongly altered in germinating vte2-1 plants (14). To examine whether this is dependent on trienoic fatty acids, each genotype (wild-type, vte2-1, fad3 fad7 fad8, and fad3 fad7 fad8 vte2) was analyzed for GST1 expression. This gene was chosen because it is strongly up-regulated in vte2-1 seedlings and because it is known to be induced by MDA treatment (10). Interestingly, the aberrant expression of GST1 that correlates with MDA overaccumulation in vte2-1 was largely abolished in the fad3 fad7 fad8 vte2 quadruple mutant. The results again show that it is trienoic fatty acid oxidation (rather than dienoic fatty acid oxidation) that correlates with and most likely underlies the vte2-1 phenotype. This phenotype is manifest throughout the body of vte2-1, and these seedlings are smaller than their wild-type counterparts (13). This raised the question of whether enhanced trienoic fatty acid oxidation leading to the production of reactive and potentially damaging molecules also takes place throughout the body of vte2-1. To address this question, it was necessary to develop an in situ assay for a product of 18:3 oxidation. MDA was chosen for this.

Development of an in Situ MDA Detection Assay—At neutral pH, MDA can exist as a charged enolate ion that is relatively stable (11). However, at pH below the pK of the enolic group (pK = 4.5), the molecule becomes protonated and reactive and can bind to nucleophilic atoms (11). This forms the basis of several assays used in MDA detection. Of these, the best known is the TBA assay, a historically important reaction between one molecule of MDA and two molecules of TBA producing the red MDA-(TBA)2 adduct that possesses a maximum light absorption (25) at 532 nm (11, 22, 23). However, this reaction is not specific, and TBA can react with other molecules, leading to interference with MDA-(TBA)2 adduct detection (24).

To develop a specific in situ method for the visualization of MDA in plant tissues using a method based on TBA, it was first necessary to have independent quantitative MDA measurements in the tissues. Herein, we quantified pentfluorophenylhydrazine derivatives of MDA according to Ref. 16. Second, a mutant used in this study (vte2-1) provided plants in which MDA levels were strongly increased relative to the wild type, and the use of the fad3 fad7 fad8 vte2 quadruple mutant demonstrated the origin of the increased levels of MDA in vte2-1 from trienoic fatty acids. These essential controls offered a platform for using the TBA test as a means of visualizing MDA in situ.

The next consideration was how to detect the adduct in the plant samples. MDA-(TBA)2 adducts exhibit strong fluorescence (17). Using fluorescence rather than absorption to detect the MDA-(TBA)2 adduct allows a sensitive and more specific detection. However, green tissues are designed to absorb light. This and chlorophyll fluorescence in plant samples are potentially serious problems, often hindering the development of in situ detection methods. Fortunately, there is a window within which chlorophyll absorption (25) is minimal in the region of interest for detecting MDA (between 500 and 570 nm). This window was exploited, and a custom-made fluorescence filter was procured. A fourth consideration was temperature. The reaction between MDA and TBA generating MDA-(TBA)2 is often conducted at 90 °C, a temperature that could potentially exacerbate the formation of artifacts in plant samples rich in trienoic fatty acids. However, the reaction is chiefly dependent on a low pH and can be performed more slowly at lower temperatures (11, 24). We chose 37 °C as a suitable temperature for the in situ MDA assay and employed 5-h incubation times. Because the in situ assay is not intended to be quantitative, it is not necessary to ensure that adduct formation has reached completion. Finally, a control to rule out the potential production of signal due to pH changes affecting the intrinsic fluorescence of the samples was necessary. This was accomplished in control experiments by using an equimolar solution of trichloroacetic acid instead of TBA. Combining all these factors, we designed a controlled in situ MDA detection method.

MDA Pools in the Arabidopsis Seedling—Using the MDA detection method, we conducted a genetic analysis of the origin of MDA as well as its localization in situ across the whole plant body. Prior HPLC analysis revealed that only one major fluorescent species, corresponding to the MDA-(TBA)2 adduct, was present in the plants, so the fluorescence observed indicated MDA and not other molecules. As in the expanded leaves of Arabidopsis (10), the cotyledons of wild-type plants contained MDA that was derived largely from trienoic fatty acids. However, weak fluorescence from the MDA-(TBA)2 adduct was still seen in cotyledons of the fad3 fad7 fad8 triple mutant, suggesting the presence of a second pool of MDA not derived from trienoic fatty acids. In addition to confirming the existence of MDA in the fad3 fad7 fad8 triple mutant using pentfluorophenylhydrazine derivatization (Fig. 2B) and HPLC of the MDA-(TBA)2 adduct with fluorescence detection (Fig. 5C), the MDA-(TBA)2 adduct was identified by liquid chromatography/mass spectrometry of extracts from wild-type and fad3 fad7 fad8 triple mutant plants (Fig. 6). The masses obtained for this adduct and for the synthetic adduct were in accord with the literature (26). The inspection of hypocotyls and roots also revealed a discrete MDA pool concentrated at the root tips in both wild-type and fad3 fad7 fad8 triple mutant plants. Given the similar fluorescence in the root tips of the two genotypes, we conclude that the majority of this MDA pool is unlikely to be derived from trienoic fatty acids. This pool of MDA is concentrated in the meristematic (cell division) region of the root in the vicinity of the quiescent center. The root cap itself was not stained. The zone that stained for MDA was composed of relatively few small cells (perhaps <100) (see scale bars in Fig. 4). Inspection of roots from radish, rice, and maize revealed similarly small zones of MDA staining associated with primary and lateral root meristem regions (data not shown). Interestingly, cells in the quiescent center of maize roots are characterized as having a highly oxidizing intracellular environment. Unlike the majority of cells in the plant, these cells contain high levels of oxidized glutathione (27). It is possible that such environments favor the production or accumulation of MDA. The discovery of this new pool will now allow a genetic analysis of its provenance. For example, the in situ assay might provide the basis for
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a genetic screen for mutants with altered levels of MDA in root tips.

The in situ analysis showed that, in vte2-1, MDA is produced throughout the body of the seedling and is not restricted to one or both cotyledons. This correlates with the phenotype of the mutant, which is also affected throughout its whole body. (All organs of vte2-1 seedlings are smaller than those of wild-type seedlings.) However, one tissue in vte2-1 that is particularly affected is the cotyledon(s), which shows strong defects after germination (13). Fluorescence from the two cotyledons in vte2-1 is similar, implying that there is a similar amount of lipid oxidation in both cotyledons. It is thus possible that one of the cotyledons is more sensitive to reactive lipid oxidation products than the other. This hypothesis is strongly supported by the fact that the treatment of germinating wild-type seeds with exogenous MDA primarily affects one cotyledon. Notably, the results do not prove that it is MDA alone that generates the phenotype. Many other products of lipid oxidation may accumulate in the mutant (14). However, the results now show that a strong visual and molecular phenotype is due to 18:3 oxidation products, among which is MDA. The in situ method for studying MDA distribution in the multicellular eukaryote A. thaliana provides a new tool for the study of the biology of one of the most ubiquitous reactive aldehydes in nature.

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