Inducible Malondialdehyde Pools in Zones of Cell Proliferation and Developing Tissues in Arabidopsis*5

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Malondialdehyde (MDA) is a natural and widespread genotoxin. Given its potentially deleterious effects, it is of interest to establish the identities of the cell types containing this aldehyde. We used in situ chemical trapping with 2-thiobarbituric acid and mass spectrometry with a deuterated standard to characterize MDA pools in the vegetative phase in Arabidopsis thaliana. In leaves, MDA occurred predominantly in the intracellular compartment of mesophyll cells and was enriched in chloroplasts where it was derived primarily from triunsaturated fatty acids (TFAs). High levels of MDA (most of which was unbound) were found within dividing cells in the root tip cell proliferation zone. The bulk of this MDA did not originate from TFAs. We confirmed the localization of MDA in transversal root sections. In addition to MDA in proliferating cells near the root tip we found evidence for the presence of MDA in pericycle cells. Remodeling of non-TFA-derived MDA pools occurred when seedlings were infected with the fungus Botrytis cinerea. Treatment of uninfected seedlings with mediators of plant stress responses (jasmonic acid or salicylic acid) increased seedling MDA levels over 20-fold. In summary, major pools of MDA are associated with cell division foci containing stem cells. The aldehyde is pathogen-inducible in these regions and its levels are increased by cellular mediators that impact defense and growth.

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4 The abbreviations used are: MDA, malondialdehyde; SA, salicylic acid; JA, jasmonic acid; PA, piperonylic acid; TFA, triunsaturated fatty-acid; GC/MS, gas chromatography/mass spectrometry; TBA, thiobarbituric acid, TCA tri-chloroacetic acid.

5 This article contains supplemental Figs. S1–S3.

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The method serves as a complement to specific quantitative assays based on a cognate MDA internal standard: 2H2-MDA (10, 11). TBA is a nonspecific chemical reagent for MDA detection, but its specificity is enhanced by using fluorescence detection of MDA-TBA adducts (16, 17). In the present work we first
conducted a quantitative analysis of MDA pools throughout the vegetative body of Arabidopsis. We then used in situ MDA detection and genetic analyses to localize major pools of MDA at the cellular and subcellular levels in leaves, in seedlings, and in the roots of seedlings. This revealed several cell types associated with high MDA levels but the largest pools of the aldehyde in the plant body are found in mesophyll cell chloroplasts (where a high proportion of MDA originates from TFAs). We show that the root proliferation zone (meristem) contains intracellular MDA most of which is not derived from TFAs. We also detected MDA in the pericycle, a cell type that retains its capacity to proliferate.

EXPERIMENTAL PROCEDURES

Plant Growth Conditions, Genotypes, and Chemicals—Wild-type (WT) Arabidopsis (Col-0), fad3–2 fad 7–2 fad8 (18), nar1 (19), npr1 (20), and peroxisome targeting sequence1 green fluorescent protein: (PTS1:GFP) expressing plants (21) were grown on soil at 22 °C for 5 weeks (light: 100 μmol m−2 s−1, 9 h light/15 h dark), or on 0.7% agar (w/v, in water) for 4 d (15 h light/9 h dark). Chemicals were used purchased from Sigma unless indicated.

Organelle Purification and Characterization—Chloroplasts were purified as described (22) and chlorophyll was determined by spectrophotometry (23). Mitochondria were enriched by differential centrifugation (24). The mitochondrial pellet was resuspended in buffer (20 mM Tricine/KOH pH 7.6, 2.5 mM EDTA, 5 mM MgCl2, 300 mM sorbitol). Proteins were precipitated at 2 °C using trichloroacetic acid (TCA, 8% w/v), quantified with a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Waltham, MA), and separated by electrophoresis on SDS-PAGE gels (12% w/v acrylamide). Blotting was conducted using a semi-dry blotter (Bio-Rad) and protein loading was evaluated by staining with Ponceau red (1% v/v). For the detection of proteins with antibodies, the membrane (Trans-Blot, Bio-Rad) was incubated with American Biosciences ECL Plus™ Western blotting Detection Reagents for 2 min followed by phosphorimaging with an ImageQuant (GE Healthcare, Little Chalfont, United Kingdom). Antibodies were against gamma tonoplast intrinsic protein (γ-TIP) (25), catalase (21), lipoxygenase 2 (AtLOX2) (26), and voltage-dependent anion channel1 (VDAC1) (27). Nuclei and damaged chloroplasts stain with DAPI (4′,6-diamidino-2-phenylindole, 0.1 μg ml−1 for 0.5 h on ice) and their number per non-stained chloroplasts was assessed by microscopy from PTS1:GFP-expressing plants.

2-Thiobarbituric Acid (TBA) Staining—Leaves (still attached to the plant) were soaked in 35 mM TBA solution and vacuum infiltrated (3 times 10 s). Next, samples were incubated for 60 min at 35 °C and fluorescence monitored by confocal microscopy (Leica SP2 confocal microscope). Extracted chloroplasts were incubated in 35 mM TBA (or 35 mM TCA as control) for 1 h at 35 °C in the dark, and the fluorescence was monitored. Seedlings were grown at 22 °C for 4 days on 0.7% agar (w/v, in water) and transferred into 35 mM TBA or TCA solutions and incubated at 25 °C for 90 min prior to microscopy. MDA-TBA adduct emission: 555 ± 15 nm, excitation 515 nm; autofluorescence of chloroplasts: emission 664–696 nm, excitation: 488 nm.

Gas Chromatography/Mass Spectrometry (GC/MS)—Quantification based on a specific pentafluorophenylhydrazine (PPFH)-MDA adduct was used with (2H3)-MDA as an internal standard (IS) (10). Plant material (about 150 mg) was harvested, frozen in liquid nitrogen, and ground to a fine powder. The frozen powder was added to PBS containing 1 mM deferoxamine mesylate, 50 μM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 50 pmol IS and 6.6 N H2SO4 (10 μl) and incubated for 10 min on ice, in the dark. Sodium tungstate (0.3 M, 75 μl) was then added and the samples centrifuged to precipitate protein (10). The supernatant was combined with buffer (0.21 M citric acid, 0.58 M Na2HPO4; pH 4) and 50 μl of PPFH reagent (5 mg ml−1 in water) and incubated for 3 h at 22 °C. 9 μl of 9 N H2SO4 was added, and the MDA-PPFH product was extracted with 125 μl of isooctane. The organic phase was recovered, and 1 μl of a 1:5 dilution was analyzed by GC/MS in electron ionization (EI) mode with split-injection (1:50), except for MDA analysis in the extracted chloroplast samples (splitless mode, no dilution, chemical ionization using methane). The temperature gradient was 50 °C for 1 min, 50–150 °C at 10 °C min−1 and 150–280 °C at 20 °C min−1. Quantification was carried out using selective ion monitoring measuring m/z 234 for MDA and m/z 236 for the internal standard.

Root Sectioning—Seedlings were grown at 22 °C for 4 d on 0.7% agar (w/v in water) plates and transferred into 35 mM TBA or TCA solutions, incubated at 25 °C for 90 min, washed with 50 mM potassium phosphate buffer (pH 6) and incubated with Calcoflour White (0.1% w/v) and Evan’s Blue (0.05% w/v) for 5 min. Plants were then washed as before and vacuum-infiltrated (3 × 15 min each) with 4% (w/v) paraformaldehyde in buffer (50 mM PIPES/KOH pH 6.5, 5 mM EGTA, 5 mM MgSO4). Seedlings were then embedded in agarose (10% w/v) and sectioned (50 μm) with a Vibratome (Leica VT1000S). Samples were visualized using a DM5000 Leica epifluorescence microscope with a YFP (yellow fluorescent protein) filter for MDA(TBA)2 and/or a blue filter for cell wall fluorescence (Leica filter cube A4 : excitation 400–420 nm, emission 430–510 nm). Confocal microscopy and comparison of emission spectra for TBA–MDA adducts and root tips treated with TBA used the Leica SP2 microscope. Piperonylic acid (PA) was dissolved in ethanol (10 mM stock solution in ethanol) and diluted 1:1000 in water prior incubation (16 h at 22 °C) of seedlings.

Infection with Botrytis cinerea and Other Treatments—Conidia from the fungus grown on 0.5x potato dextrose agar (13) were filtered and diluted to 106 spores ml−1 in 0.5× potato dextrose broth (PDB) and stored at −80 °C prior application (28). Seedlings were grown in water in continuous light for 2 days post-germination. The water was replaced by 5 μl of fungal spore suspension or with 0.5× PDB for the controls. To look at the root tip cell proliferation region in more detail we treated 4 day-old WT and fad3–2 fad7–2 fad8 seedlings with JA (50 μM) or with SA (250 μM) for 15–18 h prior to TBA treatment.
RESULTS

The quantitative GC/MS method developed by Yeo et al. (10) was used to measure MDA. A potential complication during MDA extraction is the formation of reactive oxygen species to cause lipid oxidation, a potential source of MDA (14, 17). We sought to minimize this and found that replacing the butylated hydroxy toluene from (10) with Trolox-reduced MDA production in vitro, a known phenomenon with plant tissues (15). Also, the procedure was conducted under low light and on ice. Total and free MDA in the leaves of adult plants and in whole 4-day-old seedlings was measured, as were MDA levels in the dissected roots of seedlings. The use of the fad3–2 fad7–2 fad8 mutant (18) enabled us to quantify the proportion of MDA that originates from TFAs in each of these tissues. The TBA staining method (12) was used to identify cell types containing MDA and to look for inducibility in MDA pools.

Adult Leaves—Levels of MDA in expanded Arabidopsis leaves were 33.01 ± 1.63 nmol g⁻¹ dry weight (D.W.) for the WT and 13.74 ± 0.70 nmol g⁻¹ D.W. for the fad3–2 fad7–2 fad8 mutant (Fig. 1A). These values indicate that ~58% of total MDA in resting leaves derives from the oxidation of TFAs. Omitting incubation with 6.6 N H₂SO₄ during the MDA extraction protocol allowed us to discriminate between the free and bound pools of MDA (10). An estimated 25.02 ± 2.03 nmol g⁻¹ D.W. of the MDA in WT and 3.02 ± 0.39 nmol g⁻¹ D.W. in fad3–2 fad7–2 fad8 leaves was found to occur in its free form. This represents 76% of total MDA in WT leaves and 22% of total MDA in the fad3–2 fad7–2 fad8 mutant. Quantities of bound MDA were estimated to be similar in both genotypes.

Whole Seedlings—Levels of bound and free MDA pools in 4-day-old seedlings were established (Fig. 1B). Non-TFA-derived MDA represents the majority in whole seedlings, being responsible for 65% of the total MDA. In WT seedlings, the percentage of free to total MDA was 79%, similar to the results obtained in WT leaves. The ratio for fad3–2 fad7–2 fad8 seedlings on the other hand was 80% of free MDA, compared with 22% of free MDA in the leaves.

Seedling Roots—Putative pools of MDA in the roots of both the WT and fad3–2 fad7–2 fad8 seedlings were reported (12). Here we used quantitative MDA measurements to validate these initial observations. Roots were dissected off 4-day-old seedlings just below the hypocotyl (Fig. 1C). Approximately 50 mg of D.W. of roots (equal between 1500–2000 roots) were used for each data point and this was replicated five times to provide an average value. The total root MDA levels were 3.18 ± 0.20 nmol g⁻¹ D.W. and 2.47 ± 0.03 nmol g⁻¹ D.W. for the WT and fad3–2 fad7–2 fad8, respectively. In contrast to total MDA levels in leaves and whole seedlings, levels in isolated roots were very similar between WT and fad3–2 fad7–2 fad8 and only 22% (p = 0.01) of the MDA in the root was found to be derived from TFAs. The ratio of the free MDA pool with respect to the total MDA in WT roots was approximately the same as in leaves or whole seedlings (~80%).

Subcellular Localization of MDA in Leaves—There is little information on the localization of MDA in plants although there are reports that it is found in chloroplasts in heat-stressed plants (e.g. 29). To localize MDA, expanded leaves were infiltrated with 35 mM TBA (or with 35 mM TCA as negative control) and fluorescence was observed using confocal microscopy (Fig. 2). As expected, both the TCA control and the TBA-treated samples showed chloroplast autofluorescence. However, only in the TBA-incubated sample was green fluorescence

FIGURE 1. Scale and origin of MDA pools. Bound and free MDA pools in adult leaves and 4-day seedlings of WT and fad3–2 fad7–2 fad8 (fadt) were quantified by GC/MS. Acid hydrolysis prior to derivatization was used to measure total MDA and omitted for measurement of unbound MDA. A, rosette leaves; 8 biological replicates; B, whole seedlings; 8 replicates; C, seedling roots; 5–8 replicates. D, overview of results. *, p < 0.05; **, p < 0.001; n.s., not significant.

trated with 35 mM TBA (or with 35 mM TCA as negative control) and fluorescence was observed using confocal microscopy (Fig. 2). As expected, both the TCA control and the TBA-treated samples showed chloroplast autofluorescence. However, only in the TBA-incubated sample was green fluorescence
clearly visible. The autofluorescence and the green TBA-associated fluorescence of the chloroplasts colocalized well, although we noted stronger fluorescence in the chloroplast regions proximal to the tonoplast (arrowheads in Fig. 2). During these experiments we noted that TBA-generated fluorescence was weak in the epidermis except in stomata (not shown).

Isolation of Chloroplasts—Intact chloroplasts were purified from leaves and DAPI staining allowed us to estimate a maximum contamination with 0.50% ± 0.36% S.D. of nuclei and broken chloroplasts. The analysis of chloroplast isolates from plants expressing a fluorescent peroxisome marker (21) indicated that a maximum of 1.5% of contaminating organelles were peroxisomes. Next, a series of organelle-specific antigens was chosen for immunoblot analysis of chloroplast fractions (Fig. 3A). LOX2 protein bands were visible in both intact chloroplasts and, as expected, in the crude extract which was rich in chlorophyll and broken chloroplasts. Anti-γ-TIP antibody (25) was used to evaluate contamination by tonoplasts and the lack of this band in the chloroplast sample indicates a lack of tonoplast contamination. An anti-catalase antibody (21) was used as a second test for peroxisome contamination; the results were negative. Finally, immunodetection of the mitochondrial-specific marker protein VDAC1 (27) revealed a weak band in the positive control and no band in the chloroplast fraction. We extended this investigation by using an enrichment for mitochondria to provide a second positive control to verify the proper functioning of the antibody. Only the mitochondrion-enriched sample showed a clear band for the VDAC1 protein.

Characterization of Chloroplastic MDA—Isolated chloroplasts were stained with TBA to visualize MDA (Fig. 3B). Again, faint autofluorescence was observed in the TCA control reflecting the observation made during in situ MDA staining (Fig. 2). However, the WT TBA-treated sample showed a strong green fluorescence although the asymmetry of the TBA staining seen in situ (Fig. 2) was lost in isolated chloroplasts. The fluorescence in TBA-treated fad3–2 fad7–2 fad8 chloroplast isolates appeared much reduced compared with the WT isolates indicating the presence of residual MDA pools in fad3–2 fad7–2 fad8 chloroplasts. WT chloroplasts were found to contain 3.50 ± 1.10 nmol MDA mol⁻¹ of chlorophyll and fad3–2 fad7–2 fad8 1.50 ± 0.85 nmol MDA mol⁻¹ of chlorophyll (Fig. 3C) and this difference was significant (p = 4.6 × 10⁻⁶). MDA levels in the fad3–2 fad7–2 fad8 chloroplasts represent 42% of MDA levels in the WT.

Localization of MDA in Roots—Roots have very different optical properties to leaves, so TBA staining of roots was opt-
mized by testing various incubation times (30 min to 5 h) and temperatures (20 to 37 °C). The incubation of seedlings with 35 mM TBA at 25 °C for 90 min resulted in good visualization of the putative MDA pools in roots. Consistent with Mène-Saffrané et al. (12) we found strong TBA-dependent fluorescence near the root tips and there was little or no difference in the intensity of the fluorescence between the two genotypes. WT roots were treated with TBA (or TCA as a control) and then sectioned (Fig. 4). No strong autofluorescence signal was observed in roots incubated with TCA (supplemental Fig. S1). TBA-dependent fluorescence in the proliferation region localized inside the undifferentiated meristematic cells (Fig. 4B). In contrast, in the sections of the elongation zone TBA-dependent fluorescence had a complex pattern with staining falling into two categories. Firstly, diffuse staining was seen in the stele and in particular in the pericycle and extending into other smaller cells in the stele (Fig. 4C). However, with the exception of casparian strips, the endodermis was not stained. A second type of staining pattern in the elongation zone was less diffuse. This was always associated with cell surfaces and included the protoxylem walls (supplemental Fig. S1), casparian strips, and the outer face of epidermal cells. With the exception of the casparian strips, this staining co-localized with the Calcofluor cell wall stain. Both xylem and casparian strips contain phenolic material so we used the lignin synthesis inhibitor PA (30) to investigate a possible association of TBA staining and lignification. Treatment of 4-day-old WT seedlings for 18 h with PA (10 μM) did not affect the overall TBA-staining pattern seen in the plants (not shown). Comparison of the emission spectrum of synthetic MDA-TBA adducts with that of TBA-stained root cells under closely similar conditions in a confocal microscope yielded similar but not identical spectra (Fig. 5A). Specifically, the tissue stacks displayed both an emission maximum near 550 nm (also seen with synthetic TBA-MDA adducts) as well as a prominent shoulder near 560 nm. We further examined the tips of WT roots with confocal microscopy. Using DAPI staining we were able to localize nuclei in outer cells near the root tip. In contrast, fluorescence due TBA treatment of tissues was diffuse. This green fluorescence was observed in the nuclei in some cells near the cortex but was far weaker in cells in the outermost cell layers (Fig. 5B).

MDA Dynamics during Gray Mold Infection—As the fungus grew we observed changes in TBA-dependent fluorescence in the seedlings (Fig. 6A). An apparent increase of MDA in the cotyledons, hypocotyl region, and apical meristem (leaf primordia) could be observed for both WT and fad3–2 fad7–2 fad8 seedlings. On the other hand, MDA labeling disappeared in necrotic tissue, as observed in the root tips after 40 h. Both genotypes were heavily infected showing necroses and chloroplast bleaching although there was a strong increase in MDA fluorescence in the cotyledons. Both jasmonic acid (JA) and salicylic acid (SA) are known to accumulate in this pathosystem (31). We first treated 4-day-old seedlings for 18 h with JA (50 μM). This led to changes in TBA-dependent fluorescence throughout the seedling with the strongest effect on the cotyledons and also on the apical meristem and root tip (supplemental Fig. S2A). TBA-associated fluorescence was strongly induced by treatment with SA or JA in both the WT and the fad3–2 fad7–2 fad8 root tips (Fig. 6B). Control seedlings were found to contain 3.92 ± 0.64 nmol g⁻¹ D.W. MDA. Upon treatment of the WT seedlings the amount of MDA increased to
89.02 ± 12.17 nmol g⁻¹ D.W. and 101.20 ± 27.91 nmol g⁻¹ D.W. for SA and JA, respectively (Fig. 6C). We tested whether mutants known to block elements of salicylate and jasmonate signaling interfered with MDA induction in root tips and found that jar1 blocked MDA induction by JA but that npr1 did not block MDA induction by SA (supplemental Fig. S2B). Finally, we observed TBA-dependent fluorescence in leaf primordia in unstimulated seedlings (supplemental Fig. S3A).

**DISCUSSION**

**Generalities**—TBA staining is a non-quantitative and potentially non-specific method that must be supported with analytical data based on the use of an appropriate internal standard and, if possible, with reference mutants or else using conditions

![Figure 5](image_url)  
**Figure 5.** Characterization of MDA-TBA adducts in the root tip. A, emission spectra of soluble TBA-MDA adducts produced in solution (synthetic adduct) and in situ stained root tips. Data sets were gathered under identical conditions using confocal fluorescence microscopy. B, confocal images of the root tip stained with DAPI to detect nuclei (cyan) and with TBA to detect MDA (green). The arrowhead shows a nucleus that showed TBA-related fluorescence.

![Figure 6](image_url)  
**Figure 6.** MDA pools in seedlings respond in pathogenesis and to plant-derived stress mediators. A, MDA staining in the WT and fad3–2 fad7–2 fad8 (fad7) Arabidopsis seedlings after infection. Upper panel, white light. Lower panel, TBA staining after infection with B. cinerea (40 h post-infection with 5 μl containing 10⁶ spores ml⁻¹). Arrowheads: root tips. Note that root tips have died in infected seedlings and TBA staining in this region is no longer seen. Arrows: MDA accumulation after infection in the shoot apex, hypocotyls, and cotyledons. Scale bar, 50 μm. B, TBA staining of MDA pools in root tips of WT and fad seedlings treated with JA or SA. Note lack of staining in the extremity of the root, the root cap region. Scale bar, 50 μm. C, quantitative analysis of MDA levels in WT seedlings after treatment with JA (50 μM) or SA (250 μM) 18 h at 22 °C prior to harvest. **, *p < 0.001.
that affect MDA levels. Previous mass spectral analysis has detected the presence of MDA-TBA$_2$ adducts in TBA-treated seedlings (12). The findings herein suggest that it is unbound MDA that reacts with TBA to produce this fluorescence since free pools of MDA in WT root tissue are much larger than those of bound MDA. Furthermore, the concentrations of TBA (35 mM) used, combined with mild incubation conditions (25 °C for 90 min for roots) are unlikely to release bound MDA efficiently (10, 14). As we show herein, overall TBA staining correlates with quantitative MDA measurements. Nevertheless, the fluorescence emission spectrum of TBA-stained root tip cells was more complex than that of synthetic MDA-TBA adducts, which were similar to previously published spectra (e.g. 17) but with a slight shoulder near 560 nm. This shoulder was more dominant in TBA-stained root tips. We conclude that the spectrum of fluorescent TBA adducts made in situ is likely to contain multiple species including (TBA)$_2$-MDA. This underscores the importance of quantitative assays to confirm MDA presence and abundance in biological samples.

In terms of total MDA per unit of dry mass, there is roughly an order of magnitude more of this aldehyde in leaves than in seedlings or seedling roots. Levels of unbound MDA ranged from ~2 nmol g$^{-1}$ D.W. in seedlings and isolated roots to 25 nmol g$^{-1}$ D.W. in leaves of adult plants, as measured by performing quantitative analyses in the absence of strong acid hydrolysis. In contrast, pools of bound recoverable MDA throughout the vegetative body of Arabidopsis are, in general, low. We regard our estimates for the size of these bound pools as approximate; they are derived by subtracting free from total MDA pools. The bound pools ranged from 0.59 nmol g$^{-1}$ D.W. in seedlings to 8.00 nmol g$^{-1}$ D.W. in the leaves of adult plants. Here, again there is a correlation between higher bound MDA pools and the presence of expanded leaves.

Another generality to emerge from our data is that the fad3–2 fad7–2 fad8 mutation had a high impact on total MDA levels in leaves and a low impact on total MDA pools in roots. Overall estimates for total, bound and unbound MDA in leaves support data published previously. However, the present estimate for how much MDA in leaves is derived from TFAs (58%) is lower than a previous estimate of 76% (13). This difference may be due to better sample protection in the present analysis. Unexpectedly, the fad3–2 fad7–2 fad8 mutant has differential effects on free and bound pools depending on the plant stage. The most extreme example of this is in roots where almost all free MDA is derived from unknown sources, that is, not from TFAs.

**MDA Localization in the Leaf**—TBA staining and confocal microscopy was used to localize MDA pools at the subcellular level. Shown in Fig. 2 are mesophyll cells, and we noted that chloroplasts in these cells appeared to be the main sites of MDA accumulation in the leaf. We did not observe TBA staining in vacuoles suggesting at least some specificity in the subcellular localization of unbound MDA. TBA-infiltrated leaves occasionally show some staining associated with structures estimated to be less that 1 μm in diameter, so plastids may not be the only organelles to harbor the aldehyde. During these experiments we did not detect strong fluorescence in epidermal pavement cells, but we noted that stomata fluoresced after TBA (but not TCA) treatment. Pavement cells do not contain chloroplasts, whereas stomata do. Based on this, and on the observation of putative pools of MDA in mesophyll cell chloroplasts (Fig. 2), these organelles were purified from leaves, characterized rigorously, and found to contain MDA.

Consistent with chloroplasts being an important source of MDA, the analysis of isolated chloroplasts revealed that they contained the same ratio of TFA-derived MDA (58%) as whole leaves. Previous publications have suggested that MDA is present in plastids (e.g. Ref. 29) but until now this has not been shown with rigorously purified organelles or with quantitative assays based on cognate internal standards. It is of interest that the site of MDA localization, the chloroplast stroma, contains DNA, RNA, and ribosomes. If MDA has genotoxic or proteotoxic effects in leaves these effects might be manifested on the chloroplast genome and proteome. Alternatively, and as suggested previously, MDA might act to regulate stress-related gene expression in expanded leaves (15).

**Seedling- and Root-associated MDA Pools**—Most fluorescence in the root proliferation (meristematic) zone appeared to be intracellular in undifferentiated cells. This was diffuse staining as would be expected for a low molecule mass adduct formed between TBA and MDA (14, 17), adducts that we have detected previously by mass spectrometry in TBA-treated seedlings (12). We could not identify TBA labeled subcellular compartments in root cells, although while we noted that nuclei in the root cap region did not stain strongly for MDA some nuclei in the outer layers of the proliferation zone did. It is possible that this was due to TBA-MDA adduct diffusion either into nuclei or, in outer cell layers, loss of adducts into the bathing solution. Diffuse TBA staining was also seen in the pericycle, a cell type that continues to proliferate after leaving the meristem and that gives rise to lateral roots (32). Consistent with this we have observed strong TBA-dependent fluorescence in lateral root primordia (not shown). We note that the stele contains other vascular system progenitor cells ( cambium) between the paired xylem and phloem poles. This region was also stained with TBA but it was not possible to resolve the identity of any of the stained cell types. A commonality between the pericycle and the root tip meristematic region is that they both contain stem cells. Complicating the analysis of the root was the punctuate TBA-dependent staining seen most strongly in the paired protoxylem poles, caspian strips, and the outer epidermal cell walls, and more weakly elsewhere in or near cell walls throughout the plant. We interpret this non-diffuse staining as being possibly artifactual. For example, it is conceivable that single molecules of TBA react with endogenous molecules at these sites to create tethered fluorescent molecules. The fact that a lignin synthesis inhibitor (PA) did not affect the overall staining pattern of the seedlings suggests that most of the MDA is not lignin-derived.

The root tip cell proliferation zone can be calculated to be about 0.004 mm$^2$ in volume (see scale bar in Fig. 6B) making it difficult to analyze biochemically and it is noteworthy that, without TBA staining, MDA in this zone would not have been discovered. Even for MDA quantitation in detached whole roots it was necessary to analyze between 7500 and 10,000 individual roots per replicated measurement. Our results suggest
that TBA staining could be used to search elsewhere for putative MDA pools and also to search for remodeling of MDA pools even in the absence of overall concentration changes within whole organisms. With this in mind we analyzed TBA-dependent fluorescence in seedlings infected with the fungus *B. cinerea*.

**MDA Dynamics**—There are reports that MDA levels increase modestly (i.e. not more than 2-fold) in expanded leaves exposed to heat stress or hypoxia (15) and other works suggest that MDA levels in leaves sometimes decrease in pathogenesis (15, 33). In contrast, when we infected seedlings with *B. cinerea* MDA was found to be highly inducible in cotyledons, apical meristems and root meristems. These tissues are all preformed in the embryo. However, we could not distinguish whether the plant or the fungus produced this MDA. In an attempt to resolve this we grew plants in the absence of the fungus and treated them with two mediators known to accumulate in this pathosystem (SA and JA, Ref. 31). This resulted in ~20-fold increases in MDA levels in the seedlings, allowing us to correlate increases in MDA with increases in TBA-dependent staining. The correlation between staining pattern and MDA level validated the staining method for the root, an organ for which we have yet to find a mutant that strongly impacts resting MDA levels. Moreover, the results now show that certain MDA pools associated with proliferating and embryonic cells are highly responsive to biological mediators involved in stress responses. That is, there is an association between MDA inducibility and growth potential. The JAR1-dependent and NPR1-independent induction of MDA by JA and SA, respectively is interesting since elevated levels of both mediators are known to inhibit plant growth (34, 35).

In conclusion, MDA pools in young, dividing tissues appear to be more highly inducible than their counterparts in expanded leaves. It is curious that MDA, a known genotoxin, is both present and inducible within intracellular compartments in stem cell-rich regions and roles in protecting these regions against biological invasion are plausible. The staining method should be applicable to other organisms provided that appropriate controls are used.

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