Malonylation of Glucosylated N-Lauroylethanolamine

A NEW PATHWAY THAT DETERMINES N-ACYLETHANOLAMINE METABOLIC FATE IN PLANTS

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N-Acylethanolamines (NAEs) are bioactive fatty acid derivatives present in trace amounts in many eukaryotes. Although NAEs have signaling and physiological roles in animals, little is known about their metabolic fate in plants. Our previous microarray analyses showed that inhibition of Arabidopsis thaliana seedling growth by exogenous N-lauroylethanolamine (NAE 12:0) was accompanied by the differential expression of multiple genes encoding small molecule-modifying enzymes. We focused on the gene At65g39050, which encodes a phenolic glucoside malonyltransferase 1 (PMAT1), to better understand the biological significance of NAE 12:0-induced gene expression changes. PMAT1 expression was induced 3–5-fold by exogenous NAE 12:0. PMAT1 knockouts (pmat1) had reduced sensitivity to the growth-inhibitory effects of NAE 12:0 compared with wild type leading to the hypothesis that PMAT1 might be a previously uncharacterized regulator of NAE metabolism in plants. To test this hypothesis, metabolic profiling of wild-type and pmat1 seedlings treated with NAE 12:0 was conducted. Wild-type seedlings treated with NAE 12:0 accumulated glucosylated and malonylated forms of this NAE species, and structures were confirmed using nuclear magnetic resonance (NMR) spectroscopy. By contrast, only the peak corresponding to NAE 12:0-glucoside was detected in pmat1. Recombinant PMAT1 catalyzed the reaction converting NAE 12:0-glucoside to NAE 12:0:mono- or -imalonylglicosides providing direct evidence that this enzyme is involved in NAE 12:0-glucose malonylation. Taken together, our results indicate that glucosylation of NAE 12:0 by a yet to be determined glucosyltransferase and its subsequent malonylation by PMAT1 could represent a mechanism for modulating the biological activities of NAEs in plants.

N-Acylethanolamines (NAEs)\(^2\) represent a group of conserved lipid amides that have diverse biological functions in many eukaryotes. Their basic structure consists of an ethanolamine head linked to a fatty acid tail via an amide bond (1, 2). In vertebrates, NAEs are best known for their role in the endocannabinoid signaling pathway where they function as endogenous ligands to G protein-coupled cannabinoid (CB) receptors. Binding of NAEs to CB receptors triggers a series of signaling cascades that modulate a plethora of behavioral and physiological processes such as appetite, mood, cardiovascular function, pain sensation, sleep, and reproduction (3–5).

NAEs in organisms where they have been detected consist of several molecular species, which are classified based on the number of carbons and double bonds in their fatty acid chain. For example, one of the most studied NAE species in animal systems with CB receptor-dependent functions is N-arachidonylethanolamine or anandamide. Anandamide is also known as NAE 20:4 because it has 20 carbons and 4 double bonds in its fatty acyl chain (6, 7). Although the presence of NAEs in plants has been recognized for many years, their role in plant physiological processes is limited (2, 8).

The first evidence that NAEs might have biological functions in plants came from studies of endogenous NAE levels in desiccated cotton seeds. The endogenous levels of NAEs were shown to be highest in seeds, but they were rapidly depleted during seed imbibition. This observation led to the hypothesis that NAEs might be involved in germination and early seedling establishment possibly as negative growth regulators (6, 8). In support of this notion was the observation that Arabidopsis seeds germinated on exogenous N-lauroylethanolamine (NAE 12:0) led to stunted seedling development due in part to an altered cytoskeleton and endomembrane system (9, 10). Furthermore, young Arabidopsis seedlings exposed to N-linoleylethanolamide (NAE 18:2) and N-linolenylethanolamine (NAE 18:3) had disrupted root and chloroplast development, respectively, suggesting that NAEs collectively blocked crucial

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\(^{\text{2}}\) The abbreviations used are: NAE, N-acylethanolamine; CB, cannabinoid; UHPLC, ultra-high performance liquid chromatography; ESI, electrospray ionization; QTOF, quadrupole time of flight; SPE, solid phase extraction; ANOVA, analysis of variance; qRT, quantitative RT; FAAH, fatty acid amide hydrolase; F, forward; R, reverse; ABA, abscisic acid; LOX, lipoxygenase.
processes that are part of the transition from germinative to post-germinative seedling growth (11, 12). The potential role of NAEs in seed germination and early seedling development was further reinforced by transcriptomic studies of NAE 12:0-treated seedlings, which showed that genes related to abscisic acid (ABA) signaling were differentially regulated (13, 14). It was also reported that NAE could act synergistically with ABA in inhibiting seed germination and seedling root development with the latter process regulated by the oxidative metabolism of NAE 18:2 (12, 13).

One mechanism by which the signaling and/or biological activities of NAE are modulated is through the action of the fatty acid amide hydrolase (FAAH) enzyme. FAAH hydrolyzes NAE at its amide linkage to yield an ethanolamine group and its corresponding free fatty acid (15, 16). The critical role of FAAH in regulating NAE signaling in mammals was demonstrated through a series of studies with mouse FAAH knock-out mutants. Mouse mutants with the FAAH gene inactivated resulted in the accumulation of endogenous NAEs in the central nervous system, which subsequently led to a decrease in sensitivity to pain and increased sensitivity to anandamide (17, 18). The response of plants to NAE has also been shown to change when the expression level of a plant’s FAAH orthologue is modified. For example, knockouts to Arabidopsis FAAH (faah) were more sensitive to the growth-inhibitory effects of NAE 12:0, whereas FAAH overexpressors were less sensitive to NAE 12:0-induced growth arrest (19). The observation that FAAH-altered plants had modified endogenous NAEs (i.e. faah had higher NAEs whereas FAAH overexpressors had lower NAEs) is consistent with FAAH’s function as an important enzyme involved in plant NAE hydrolysis in vivo (19, 20). Recently, the formation of bioactive NAE oxylipin metabolites from NAE 18:2 and NAE 18:3 in plants that disrupt root and chloroplast development was shown to be catalyzed by lipoygenase (LOX) enzymes (11, 12). The LOX-mediated oxidation of polyunsaturated NAEs in plants is reminiscent of the eicosanoids, a class of mammalian hormones that are oxidation products of cyclooxygenase and LOX enzymes (21). Information on other enzymes that regulate NAE levels in plants has remained elusive.

To discover new players involved in regulating the metabolic fate of NAE in plants, we revisited our previous transcriptomic data of seedlings treated with NAE 12:0. In addition to ABA-related transcripts, genes encoding enzymes that modify low molecular mass compounds were up-regulated in seedlings exposed to NAE 12:0 (13). Modification of bioactive small molecules is a typical way of removing or inactivating potentially damaging compounds so the plant can protect itself (22–25). Alternatively, small molecule modification could be a mechanism by which its signaling activity is regulated. Evidence for the latter comes from studies of the hormones, auxin and jasmonic acid. Both auxin and jasmonic acid are conjugated into biologically inactive forms by specific enzymes to dampen their activity within the cell (26, 27). Conjugation of bioactive small molecules not only leads to their inactivation but also to their preferential transport to cellular compartments or to the extracellular matrix where they are spatially separated from their biological targets (28). In this regard, it is possible that some of the genes encoding small molecule-modifying enzymes up-regulated by NAE 12:0 could function as NAE-modifying enzymes. Here, we present genetic and biochemical evidence that phenolic glucoside malonyltransferase 1 (PMAT1), which was previously shown to have malonyltransferase activity to a range of phenolic glucosides (25), has a similar effect on NAE 12:0 glucosides. PMAT1 catalyzes the reaction converting glucosylated NAE 12:0 to NAE 12:0 malonylglucosides. We propose that the formation of an NAE glucoside and its subsequent malonylation by PMAT1 likely represents a pathway for the storage and/or metabolic inactivation of NAE in plants.

**Results**

**Gene Encoding Phenolic Glucoside Malonyltransferase 1 Is Up-regulated by NAE 12:0—A** previously conducted microarray-based gene expression analysis on wild-type Arabidopsis seedlings treated with NAE 12:0 revealed the up-regulation of a number of genes encoding small molecule-modifying enzymes. Here, we focused on characterizing a gene (At5g39050) encoding PMAT1, which was shown by microarray data to be up-regulated 3-fold by NAE 12:0 treatment (13). Quantitative reverse transcriptase-PCR (qRT-PCR) revealed that PMAT1 gene expression was enhanced by about 5-fold in seedlings treated with NAE 12:0 validating the results obtained with microarrays (Fig. 1A).

PMAT1 Mutants Are Less Sensitive than Wild Type to the Growth-inhibitory Effects of NAE 12:0—Exogenous NAE 12:0 inhibits the growth of Arabidopsis seedlings (9, 10). As such, NAE 12:0-induced seedling growth inhibition has served as a convenient biological readout for evaluating pathways involved in NAE metabolism in plants using gene knockouts and overexpressors (13, 19). Following similar genetic strategies, we tested whether altered PMAT1 expression had any effect on seedling growth when exposed to exogenous NAE 12:0. We obtained mutants to PMAT1 from the Arabidopsis Biological Research Center. The Arabidopsis PMAT1 gene consists of a single exon, and we identified two mutant alleles (SALK_110268 and SALK_007564) with transfer (T)-DNA insertions within the exon. We refer to these mutants as pmat1-1 and pmat1-2 following the nomenclature of Taguchi et al. (25) (Fig. 1B; supplemental Fig. S1). Semi-quantitative RT-PCR showed that pmat1-1 and pmat1-2 were null mutants as no transcripts were detected using PMAT1 gene-specific primers (Fig. 1C).

Wild-type, pmat1-1, and pmat1-2 seedlings on liquid medium under a 14-h light, 10-h dark cycle exhibited robust growth after 10 days (Fig. 2A). When 40 μM NAE 12:0 was included in the medium, growth of all three genotypes was strongly inhibited. We observed, however, that the extent of NAE 12:0-induced growth inhibition was less in the two pmat1 mutant alleles when compared with wild type. Whereas pmat1 mutants had green cotyledons on 40 μM NAE 12:0, those of wild-type seedlings were smaller and produced no or very little chlorophyll (Fig. 2, B and C). Growth of wild-type and pmat1 seedlings was quantified by measuring primary root length and cotyledon area in semi-solid medium with or without 40 μM NAE 12:0. In solid medium without NAE 12:0, primary root length and cotyledon area of wild-type and pmat1 seedlings
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were not different from each other. By contrast, values for primary root length and cotyledon area of pmat1 seedlings on 40 μM NAE 12:0 were significantly higher compared with wild-type seedlings (Fig. 2, D and E). The ability of pmat1 to tolerate growth on exogenous NAE 12:0 could not be overcome by simply increasing the concentration of NAE 12:0 in the media. Cotyledons of light-grown pmat1 seedlings exposed to NAE 12:0 concentrations as high as 250 μM remained larger and greener compared with wild type (supplemental Fig. S2).

The extent of growth inhibition triggered by NAE 12:0 was more pronounced when seedlings were kept in total darkness. Compared with seedlings subjected to a 14-h light/10-h dark cycle, hypocotyls of seedlings kept in the dark elongate rapidly and produce no chlorophyll in their cotyledons (Fig. 2F). In the presence of exogenous NAE 12:0 and at concentrations much lower than those needed to inhibit seedling growth in the light, hypocotyl elongation of dark-grown wild-type seedlings was severely stunted. By contrast, dark-grown pmat1 seedlings on NAE 12:0 had significantly longer hypocotyls and cotyledons that expanded more compared with wild-type seedlings (Fig. 2, F and E). Taken together, our results indicate that loss of PMAT1 function rendered seedlings less sensitive to the growth-inhibitory effects of NAE 12:0.

Reduced Sensitivity of PMAT1 Seedlings to the Growth-inhibitory Effects of NAE 12:0 Is Due to Loss of PMAT1 Function—To confirm that loss of PMAT1 function is the cause for the increased tolerance of pmat1 seedlings to the growth-inhibitory effects of NAE 12:0, we expressed a 35S:PMAT1 construct in the pmat1-1 and pmat1-2 backgrounds. Expression of 35S:PMAT1 was verified by qRT-PCR analysis. Consistent with our semi-quantitative RT-PCR results (Fig. 1C), both pmat1 mutant alleles had no detectable PMAT1 transcripts. By contrast, PMAT1 transcripts were detected in two independent lines for each pmat1 mutant allele transformed with the 35S:PMAT1 construct (Fig. 3A). When pmat1-complemented seedlings were grown on exogenous NAE 12:0 in light or in darkness, they exhibited similar growth responses as wild-type seedlings (Fig. 3, B and C) indicating that loss of PMAT1 function is indeed responsible for the reduced growth sensitivity of seedlings to exogenous NAE 12:0.

We also transformed wild-type with the 35S:PMAT1 construct to determine whether PMAT1 overexpression had any effect on seedling growth with or without NAE 12:0. We selected two independent overexpressing lines that were determined by qRT-PCR to have PMAT1 expression at levels 10–15-fold more than wild type (Fig. 3A). The response of the PMAT1 overexpressors on NAE 12:0-supplemented media and their growth on media without NAE 12:0 were similar to wild type (Fig. 3, B and D).

Metabolite Profiling and Nuclear Magnetic Resonance Spectroscopy Show That NAE 12:0 Is Glucosylated and Malonylated in Arabidopsis Seedlings—To better understand the biochemical basis for the enhanced tolerance of pmat1 to exogenous NAE 12:0, global metabolite profiles were obtained from NAE 12:0-treated and non-treated (DMSO control) wild-type seedlings using ultra-high performance liquid chromatography (UHPLC) coupled to electrospray ionization (ESI)-MS. We focused our analyses on three major metabolites that were strongly induced in NAE 12:0-treated seedlings but absent in non-treated seedlings (Fig. 4A). The first metabolite (P1) had a retention time of 17.25 min and three major ions, m/z 428.265 [M + Na]+, m/z 406.280 [M + H]+, and m/z 244.225 [M + H − 162]+, corresponding to the loss of a hexose. The second metabolite (P2) had a retention time of 17.87 min and three major ions, m/z 514.259 [M + Na]+, 492.277 [M + H]+, and m/z 244.223 [M + H − 248]+, which corresponds to the loss of a malonyl hexose moiety. The third metabolite (P3) had a retention time of 18.56 min and three major ions, m/z 600.260 [M + Na]+, m/z of 578.279 [M + H]+, and m/z 244.223 [M + H − 334]+, which corresponds to a loss of a dimalonyl hexose moiety. UHPLC-PDA-MS analyses indicated that these three unknown metabolites could represent glucose and malonyl conjugation products of NAE 12:0 (Fig. 4B; supplemental Table S1).

The three unknown metabolites that accumulated in NAE 12:0-treated samples were purified using a Waters UHPLC coupled to a Bruker QTOF-MS and automated solid phase extraction (SPE). The purified analytes were then used for structural
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FIGURE 2. pmat1 seedlings exhibit reduced sensitivity to the growth-inhibitory effects of exogenous NAE 12:0. Seedlings of wild-type and pmat1 mutant alleles grown in liquid medium without (A) or with 40 μM NAE 12:0 (B and C) for 10 days are shown. Quantification of primary root length (D) and cotyledon area (E) of wild-type and pmat1 seedlings on solvent control (DMSO) and 40 μM NAE 12:0. Statistical significance in D, E, and G was determined by one-way ANOVA. Means are (n = 12–25 seedlings) ± S.E. Different letters are significantly different (p < 0.05, Tukey’s test).

FIGURE 3. Functional complementation of pmat1 with 35S:PMAT1. A, quantitative RT-PCR of 8-day-old wild type (Wt) and different PMAT1 genotypes. Two independent lines for each pmat1 mutant allele (L1 and L2) transformed with 35S:PMAT1 had 4–7-fold increased PMAT1 expression. Two independent wild-type lines transformed with 35S:PMAT1 had 10–15-fold increased PMAT1 expression. Light (B) or dark (C)-grown wild-type and pmat1 seedlings on 15 μM NAE 12:0. Statistical significance in D, E, and G was determined by one-way ANOVA. Means are (n = 12–25 seedlings) ± S.E. Different letters are significantly different (p < 0.05, Tukey’s test).
determination by NMR. NMR spectra indicated that the P1 metabolite was glucose-conjugated to NAE 12:0 at the hydroxyl position of the ethanolamine head through a glycosidic linkage. For the P2 metabolite, malonic acid was determined to be conjugated at the carbon 6 (C6) position of the glucose-NAE 12:0 molecule. The P3 metabolite had malonic acid conjugated to C6 and C3 of the glucose-NAE 12:0 molecule (Fig. 4C). Taken together, NMR spectra confirm that the exogenous NAE 12:0 is converted by seedlings into an NAE 12:0 glucoside and then malonylated at two separate positions on the glucose molecule (supplemental Figs. S3–S9).

NAE 12:0 Malonylglucosides Are Absent in PMAT1 Mutants and PMAT Has Malonyltransferase Activity toward NAE 12:0 Glucosides—As noted, PMAT1 was previously shown to exhibit malonyltransferase activity to a range of phenolic glucosides (25). Based on our UHPLC-MS-SPE-NMR analyses of Arabidopsis seedlings treated with NAE 12:0, we hypothesized that PMAT1 might also function as a malonyltransferase for NAE 12:0 glucosides. To test this hypothesis, we compared metabolite profiles of pmat1 and wild-type seedlings treated with NAE 12:0. We found that the NAE 12:0 malonylglucosides corresponding to the P2 and P3 metabolites in NAE 12:0-treated wild-type seedlings were not detected in pmat1. Furthermore, the peak area of P1, NAE 12:0 glucoside, increased in pmat1, whereas the NAE 12:0 malonyl glucoside peaks were restored in pmat1 transformed with 35S::PMAT1 (Fig. 5).

The absence of NAE 12:0 malonylglucosides in pmat1 strongly suggested that PMAT1 is responsible for the malonylation of NAE 12:0 glucoside. To obtain direct evidence that PMAT1 has NAE 12:0 glucoside malonyltransferase activity, we expressed PMAT1 in Escherichia coli and purified the recombinant protein for in vitro enzymatic studies (Fig. 6A). NAE 12:0 glucoside P1 was purified from extracts of wild-type seedlings treated with NAE 12:0 by UHPLC-QTOF-MS-SPE and used as a substrate for our enzymatic assays. Reaction with PMAT1 recombinant enzyme was initiated by the addition of the NAE 12:0 glucoside substrate. The reaction was incubated at 30 °C for 60 min and terminated by the addition of HCl.

FIGURE 4. UHPLC-MS metabolite profiling of conjugated NAE 12:0 species in Arabidopsis seedlings. A, region of the total ion chromatogram showing three metabolites (P1, P2, and P3) induced in wild-type seedlings treated with 35 μM NAE 12:0. B, positive-ion MS of the three metabolites. The loss of the hexose and malonyls can be seen. C, structures of NAE 12:0 conjugation products determined by NMR.
UHPLC-MS analyses of products from the reaction mixture revealed an abundance of the P2 product ion corresponding to NAE 12:0 mono-malonylglucoside (P2) and a corresponding depletion of NAE 12:0 glucoside (P1) indicating that that PMAT1 catalyzes the conjugation of the first malonyl to NAE 12:0 glucoside. The peak corresponding to NAE 12:0 di-malonylglucoside (P3) was not present in the products of the reaction mixture (Fig. 6).

**Discussion**

By revisiting previous global transcriptomics data of NAE 12:0-treated *Arabidopsis* seedlings (13), we present genetic and biochemical evidence for an additional pathway that determines the metabolic fate of NAE 12:0 in plants. This pathway involves the glucosylation of NAE 12:0 and subsequent malonylation of the NAE 12:0-glucoside product (Fig. 7). The accumulation of NAE 12:0-O-glucosides in wild-type *Arabidopsis* seedlings treated with exogenous NAE 12:0 is reminiscent of naphthol-glucoside formation in *Arabidopsis* plantlets exposed to elevated levels of naphthols (24, 25). Because exogenous NAE 12:0 has inhibitory effects on seedling growth (9, 10), plants could be responding to NAE 12:0 in a manner similar to that of phenolic compounds (i.e. by activating general detoxifying mechanisms). Consistent with this hypothesis is the observation that several genes encoding glucosyltransferases and glutathione S-transferases, which are enzymes that detoxify harmful small molecular compounds by conjugating them with glucose and glutathione, respectively, were up-regulated by NAE 12:0 (13, 29, 30). The glucosyltransferase(s) responsible for glucose conjugation of NAE 12:0 remains to be identified.

Taguchi et al. (25) showed that PMAT1 is a key enzyme that catalyzes the malonylation of naphthol glucosides and a range of other phenolic glucosides in *Arabidopsis*. Here, we found that two major metabolites from extracts of NAE 12:0-treated wild-type *Arabidopsis* seedlings corresponded to NAE 12:0-mono- and dimalonylglucosides. These NAE 12:0 conjugates were not detected in *pmat1* knockouts but were restored by genetic complementation with 35S:PMAT1. The depletion of malonylated NAE 12:0-O-glucosides in *pmat1* was accompanied by an increase in the peak signal intensity of NAE 12:0-O-glucoside. Furthermore, *in vitro* assays showed that recombinant PMAT1
catalyzed the formation of NAE 12:0-monomalonyl-O-glucoside using NAE 12:0-O-glucosides as substrates. Taken together, our results provide compelling evidence that PMAT1 also functions as an NAE 12:0-O-glucoside malonyltransferase.

The growth response of pmat1 mutants to exogenous NAE 12:0 mirrored their response to naphthols. Like their decreased sensitivity to naphthols (25), pmat1 seedlings were less sensitive to the growth-inhibitory effects of NAE 12:0. Malonylated glucosides of other plant secondary compounds are typically transported to the vacuole for storage (31–33). Assuming that malonylated glucosides of NAE 12:0 follow the same fate as other malonylated glucoside compounds, one would have expected pmat1 mutants to be more strongly inhibited by either NAE 12:0 or naphthols because of their reduced capacity to conjugate these compounds for sequestration to the vacuole. Taguchi et al. (25) reported that the lack of malonyl transferase activity in pmat1 knockouts led to the preferential transport of naphthol glucosides to the extracellular medium. As such, naphthols would be less damaging to cellular compartments and explain why pmat1 is better able to tolerate naphthol toxicity. A similar scenario could be occurring in NAE 12:0-treated pmat1 seedlings. In support of this notion was the observation that peak intensity of NAE 12:0 glucosides increased in pmat1, and this NAE 12:0 conjugate could also be detected in the extracellular media of both wild type and pmat1. Another possibility is that excess malonylated NAE glucosides are not efficiently transported to the vacuole in wild-type seedlings and as a result can cause more severe inhibitory effects on seedling growth than unconjugated NAE 12:0 or NAE 12:0 glucoside. In this regard, the lack of NAE 12:0 glucoside malonyltransferase activity in pmat1 could explain their partial tolerance on NAE 12:0-supplemented media. It remains to be tested whether any of the NAE 12:0 conjugation products reported here have any biological activity or whether the levels of glucosylated or malonylated derivatives correlate with the enhanced tolerance of pmat1 to exogenous NAE 12:0.

The lack of obvious phenotypes in pmat1 mutants in the absence of NAE 12:0 could indicate that the growth-inhibitory effects of NAE 12:0 are synthetic and not part of an endogenous plant developmental pathway. A similar argument could also be made from studies of another plant NAE-metabolizing enzyme where Arabidopsis faah mutants had no distinct phenotypes in NAE-free media but showed hypersensitivity to the growth-inhibitory effects of exogenous NAEs (19). The absence of phenotypes in single mutants to NAE-conjugating or -hydrolyzing enzymes could be due to functional redundancy as there are six other PMAT-like genes and seven FAAH-like genes in Arabidopsis (8, 25). Furthermore, the roles of NAE in animals encompass a wide range of biological processes (3–5). Plant phenotypes in response to ABA and bacterial pathogens have been reported but only for FAAH-overexpressing lines suggesting that these endogenous lipid mediators might also affect a variety of plant physiological processes (13, 34). For the future, it will be crucial to test whether single or combinatorial mutants to these NAE metabolic enzymes have any distinct phenotypes when subjected to range of environmental conditions.

FIGURE 7. Summary of NAE metabolism via hydrolysis, oxidation, and glucosylation-malonylation. Saturated (NAE 12:0) and polyunsaturated (NAE 18:2 and NAE 18:3) NAEs are hydrolyzed by FAAH to generate ethanolamine and free fatty acids. There is also evidence that NAE 18:2 and NAE 18:3 undergo oxidation by LOX to generate hydroperoxides. The pathway reported here shows that NAE 12:0 is glucosylated by an unknown glucosyltransferase, and the glucose-NAE 12:0 conjugate is malonylated by PMAT1.
Although our results suggest that glucosylation and malonylation of NAE 12:0 could be part of a general detoxification response of the plant, we cannot discount the possibility that the conjugation reactions reported here represent an endogenous pathway for the storage of NAE 12:0. Endogenous NAE 12:0 is typically found at very low nanomolar levels in vegetative tissues and young Arabidopsis seedlings (19). Thus, more sensitive targeted methods will need to be developed to detect endogenous NAE 12:0 conjugation products. It is only through the external application of NAE 12:0 that the endogenous conjugation machinery can be revealed. Similar approaches that involved exogenous application of NAES were used to demonstrate the existence of LOX-derived polysaturated NAE oxylipin metabolites in plants (11, 12). It is tempting to speculate that PMAT1 and a yet to be identified glucosyltransferase are pivotal components of an endogenous metabolic pathway that modulates the biological activity of NAE 12:0. To date, the only known enzymes in plants that regulate endogenous NAE levels and thus their biological activity are FAAH (35, 36) and LOX (11, 12). Whereas FAAH inactivates NAE signaling activity by breaking it down into its corresponding ethanolamine head and free fatty acid (15, 16), and LOX forms several bioactive NAE oxylipins (11, 12), the pathway we propose here involves a mechanism for endogenous NAE 12:0 storage or its conversion into forms that can be more readily transported into specific cellular compartments and among different tissues (Fig. 7).

Malonylation has been shown to stabilize flavonoids by preventing flavonoid glucoside malonates from enzymatic degradation (37, 38) and has been proposed to regulate the distribution and storage of diterpene glucosides, which could play a role in plant response to herbivory (39). Thus, in addition to NAE 12:0 storage, malonylation of NAE 12:0 glucosides might stabilize NAEs from degradation by FAAH providing another level of fine-tuning its signaling activity. For the future, it would be interesting to determine how FAAH- or LOX-based modulation of NAE levels interacts with the glucosylation-malonylation pathway we report here.

Experimental Procedures

cDNA Synthesis and qRT-PCR—To confirm our previous microarray data, we treated 5-day-old wild-type seedlings with 35 μM NAE 12:0 and the corresponding solvent control solution (DMSO) and harvested seedlings after 48 h. For qRT-PCR of PMAT1 transcripts in pmat1, pmat1(3SSApPMAT1), and wild type (3SSApPMAT1), we used 6-day-old seedlings. Total RNA was extracted from the different lines with RNeasy mini kit (Qiagen) as per the manufacturer’s instructions, quantified with a nanodrop (ND-1000), and treated with TURBO DNA-free kit (Ambion) to remove contaminating genomic DNA. cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen), and RT and qRT-PCRs were performed with PMAT1 gene-specific primers qRT-PMAT1-F, CGACCTCTTT-GAACGAAACCACAGG, and qRT-PMAT1-R, CTTCAGGGAGAAGCCCAAGCTC; and ACTIN2 control primers qRT-ACT2-F, GAGGGTTTCTCTTTCTCCTGCGTGGG; and qRT-ACT2-R, CCTCAGCCACATTCGTGATCTTCC. The qRT-PCRs were performed with an ABI-7900 HT instrument using SYBR Green Jumpstart Taq ReadyMix (Sigma). Data were normalized with ACT2, At3g18780 reference gene.

Plant Material and Seedling Phenotypic Assays—To confirm our previous microarray data, we treated 5-day-old wild-type Arabidopsis seedlings with 35 μM NAE 12:0 and the corresponding solvent control solution (DMSO) and harvested seedlings after 48 h. For qRT-PCR of PMAT1 transcripts in pmat1, pmat1(3SSApPMAT1), and wild type (3SSApPMAT1), we used 6-day-old seedlings. Total RNA was extracted from the different lines with RNeasy mini kit (Qiagen) as per the manufacturer’s instructions, quantified with a nanodrop (ND-1000), and treated with TURBO DNA-free kit (Ambion) to remove contaminating genomic DNA. cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen), and RT and qRT-PCRs were performed with PMAT1 gene-specific primers qRT-PMAT1-F, CGACCTCTTT-GAACGAAACCACAGG, and qRT-PMAT1-R, CTTCAGGGAGAAGCCCAAGCTC; and ACTIN2 control primers qRT-ACT2-F, GAGGGTTTCTCTTTCTCCTGCGTGGG; and qRT-ACT2-R, CCTCAGCCACATTCGTGATCTTCC. The qRT-PCRs were performed with an ABI-7900 HT instrument using SYBR Green Jumpstart Taq ReadyMix (Sigma). Data were normalized with ACT2, At3g18780 reference gene.

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Experimental Procedures

cDNA Synthesis and qRT-PCR—To confirm our previous microarray data, we treated 5-day-old wild-type seedlings with 35 μM NAE 12:0 and the corresponding solvent control solution (DMSO) and harvested seedlings after 48 h. For qRT-PCR of PMAT1 transcripts in pmat1, pmat1(3SSApPMAT1), and wild type (3SSApPMAT1), we used 6-day-old seedlings. Total RNA was extracted from the different lines with RNeasy mini kit (Qiagen) as per the manufacturer’s instructions, quantified with a nanodrop (ND-1000), and treated with TURBO DNA-free kit (Ambion) to remove contaminating genomic DNA. cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen), and RT and qRT-PCRs were performed with PMAT1 gene-specific primers qRT-PMAT1-F, CGACCTCTTT-GAACGAAACCACAGG, and qRT-PMAT1-R, CTTCAGGGAGAAGCCCAAGCTC; and ACTIN2 control primers qRT-ACT2-F, GAGGGTTTCTCTTTCTCCTGCGTGGG; and qRT-ACT2-R, CCTCAGCCACATTCGTGATCTTCC. The qRT-PCRs were performed with an ABI-7900 HT instrument using SYBR Green Jumpstart Taq ReadyMix (Sigma). Data were normalized with ACT2, At3g18780 reference gene.

Plant Material and Seedling Phenotypic Assays—Two T-DNA lines, SALK_110268 (pmat1-1) and SALK_007564 (pmat1-2), were obtained from the Arabidopsis Biological Resource Center, Columbus, OH, for the PMAT1 gene (At5g39050) (40). To isolate homozygous mutants, plants were grown and genotyped with the gene-specific primers PMAT1-F, CTACACGCGGAAGCTC; and qRT-PMAT1-R, CTTGAGGTTATCCCTTTC; PMAT1-R, GAGTAAAACAATCACAAAGTGG, and T-DNA primer LB1–3-ATTTTGGCGATTTTCGGAC. To generate complementation and overexpression lines, the PMAT1 gene was PCR-amplified from genomic DNA of wild-type Arabidopsis seedlings with primers PMAT1-Ncol-F, CCATGGATGGTGGAAACGAAGAATGG, and PMAT1-NotI-R, GCGGCCGCTTAATTTTCTAGTCCC. The resulting PCR product was cloned into the pGEM-T easy vector (Promega), and the vector carrying the insert was digested with Ncol and PmiI restriction enzymes (Invitrogen). The digested insert was then and cloned into a modified pCAMBIA1390 vector (41). The resulting 35S: PMAT1 construct was transformed into Agrobacterium cells and used to complement the pmat1 T-DNA lines or overexpressed in wild-type Arabidopsis.

For phenotypic assays, seeds of the different genotypes were surface-sterilized for 10 min in 30% bleach and rinsed four times with sterile deionized water. For seedling growth assays, seeds were placed in liquid or solid 0.5× Murashige and Skoog salts nutrient media supplemented with 1% sucrose in NAE 12:0 or the corresponding solvent control solution. Plates were wrapped with parafilm, stratified for 48 h in the dark at 4 °C, and then incubated in a growth room with a 14-h light/10-h light/dark cycle (100 μmol m−2 s−1) at 20–22 °C. Images for primary root length were taken at 10 days after stratification. Primary root lengths and cotyledon area were measured with ImageJ. For hypocotyl assays, seeds were plated and stratified as above, incubated in light for 12 h, and then grown vertically in the dark for 5 days.

Recombinant Protein Expression and Enzymatic Assay—For recombinant protein expression, the PMAT1 gene was PCR-amplified from wild-type Arabidopsis genomic DNA with gene-specific primers PMAT1-BamHI-F, GGATCCATGTT-GAACGAAAGAATGG, and PMAT1-Ndel-R, GCGGCCGCTTAATTTTCTAGTCCC. The resulting PCR product was digested with BamHI and Ndel restriction enzymes (Invitrogen) and then cloned into the pET32a+ vector. The resulting pET32a+(PMAT1) construct was transformed into BL21 E. coli cells (Invitrogen), and protein was expressed by growing cells at 28 °C to OD 0.6 and induced with 1 mm isopropyl β-D-1-thiogalactopyranoside for 2 h. Cells were lysed by sonication for 3 min at 30 intervals in 1× phosphate buffer, and protein was purified with nickel-nitrotriacetic acid purification kit (Invitrogen). The protein aliquots were separated by SDS-PAGE (10% resolving gels) and visualized in gels by Coomassie Blue staining. The enzymatic reaction was performed according to Taguchi et al. (25). The reaction mixture containing the PMAT1 enzyme and 200 μM malonyl-CoA in 50 mm potassium phosphate, pH 8.0, and 5 mm 2-mercaptoethanol was initiated by the addition of NAE 12:0 glucoside and incubated at 30 °C.
for 60 min. The reaction was terminated by the addition of 10 µl of 1 M HCl and injected into the HPLC after addition of the internal standard.

**Metabolite Profiling of NAE 12:0-treated Seedlings**—Seedlings of wild type and the different PMAT1 genotypes were grown for 8 days in liquid MS media supplemented with DMSO or 35 µM NAE12:0 with a 14-h light/10-h light/dark cycle (100 µmol·m⁻²·s⁻¹) at 20–22 °C. Seedlings were harvested and washed three times with deionized water to remove exogenous NAE 12:0. The media in which the seedlings were grown were also collected and lyophilized. Lyophilized samples were ground and extracted for 2 h in 80% methanol containing 20 µg/ml umbelliferone as an internal standard. Samples were centrifuged, and the supernatant was analyzed using a Waters UHPLC coupled to a WaterQToFMS Premier™. At least three replicates were analyzed for each sample. Separations were achieved using a Waters Acquity UPLC, 2.1 × 150-mm BEH C18 column, mobile phases of 0.1% aqueous acetic acid (A) and acetonitrile (B), and a linear gradient of 95:5 to 30:70% eluents A/B in 30 min, 30 to 5% A over 3.0 min, and 95 to 95% A over 3.0 min with a flow rate of 0.56 ml/min column temperature of 60 °C. The mass spectrometer was operated in positive electrospray ionization mode. Tandem mass data were acquired for targeted masses using a collision energy ramp from 55 to 110 eV. The MS system was calibrated using sodium formate, and raffinose was used as the reference compound.

UHPLC separations were performed using a Waters Acquity UPLC with a BEH C18 column (2.1 × 150 mm, 1.7-µm particles). A column flow of 500 µl/min and temperature of 60 °C was used. Solvents were water with 0.05% formic acid (A) and acetonitrile (B). The solvent gradient started at 95% A (aqueous) and decreased to 30% A in 30 min then decreased to 5% A in 3 min followed by 5% A for 3 min for a total run time of 40 min. Mass spectra were recorded on a Bruker (Billerica, MA) maXis Impact quadrupole time of flight (QTOF) mass spectrometer in positive electrospray ionization mode. A mass range of 9 ppm for 1H and 200 ppm for 13C, 2048 × 128 data points, and with a relaxation delay of 2 s and multiplied with an exponential function for a line-broadening of 0.3 Hz before Fourier transformation. Multiplicity-edited HSQC (pulse sequence, hsqc-edgtps3) spectra were acquired with spectral widths of 9 ppm for 1H and 200 ppm for 13C, 2048 × 128 data points, and with a relaxation delay of 2 s and multiplied with an exponential function for a line-broadening of 1 Hz before Fourier transformation. All NMR spectra were processed using Bruker's TopSpin 3.2 software.

**Author Contributions**—B. R. K., K. D. C., and E. B. B. conceived and designed the study. B. R. K., D. J. W., D. H., and L. W. S. acquired, analyzed, and interpreted data. All authors contributed to writing the article and approved the final version.

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