Seedling Chloroplast Responses Induced by N-Linolenoylethanolamine Require Intact G-Protein Complexes \footnote{OPEN}

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In animals, several long-chain N-acyl ethanolamines (NAEs) have been identified as endocannabinoids and are autocrine signals that operate through cell surface G-protein–coupled cannabinoid receptors. Despite the occurrence of NAEs in land plants, including nonvascular plants, their precise signaling properties and molecular targets are not well defined. Here we show that the activity of N-linolenoylethanolamine (NAE 18:3) requires an intact G-protein complex. Specifically, genetic ablation of the Gβγ dimer or loss of the full set of atypical Gα subunits strongly attenuates an NAE-18:3–induced degreening of cotyledons in Arabidopsis (Arabidopsis thaliana) seedlings. This effect involves, at least in part, transcriptional regulation of chlorophyll biosynthesis and catabolism genes. In addition, there is feedforward transcriptional control of G-protein signaling components and G-protein interactors. These results are consistent with NAE 18:3 being a lipid signaling molecule in plants with a requirement for G-proteins to mediate signal transduction, a situation similar, but not identical, to the action of NAEs in animal systems.

N-acyl ethanolamines (NAEs) are a family of functionally diverse signaling lipids, which consist of a fatty acid linked by an amide bond to ethanolamine and are classified based on the number of carbons and degree of saturation in their acyl chain (Blancaflor et al., 2014). Low levels of NAEs were identified in a mammalian brain in 1965 (Bachur et al., 1965), but the function of this family of lipids remained somewhat obscure until N-arachidonylthanolamine (anandamide; NAE 20:4) was discovered to be an endogenous ligand of plasma membrane-localized, G-protein coupled cannabinoid (CB1 and CB2) receptors (Devane et al., 1992; Felder et al., 1993). Upon receptor binding, anandamide stimulates a series of signaling events that regulate multiple physiological and behavioral processes in animals, including antinociception, energy balance, cognition, anxiety, and memory (Alger, 2004; Iannotti et al., 2016). A number of canonical signaling pathway components, such as the heterotrimeric G-protein complex, the calcium and potassium channels, and the adenylyl cyclase and mitogen-activated protein kinases are involved in this endocannabinoid signaling system (Fonseca et al., 2013). Other more abundant types of NAEs (e.g. ethanolamides of oleic, linoleic, linolenic, and palmitic acids) are also recognized for their role as lipid mediators, but they function independently of the G-protein–coupled cannabinoid receptors (LoVerme et al., 2005; Movahed et al., 2005).

NAEs in plants occur at low endogenous concentrations in all plant species examined, with the highest levels found in desiccated seeds (Chapman et al., 1999; Chapman, 2004). In most seeds tested, the majority of NAEs are NAE 18:3, NAE 18:2, NAE 18:1, and NAE 16:0, with NAE 18:2 often being the most abundant type (Chapman et al., 1999; Blancaflor et al., 2003; Chapman, 2004; Venables et al., 2005). Other types, such as NAE 12:0, NAE 14:0, and NAE 18:0, are also present but at lower amounts, and vary from species to species (Chapman et al., 1999; Blancaflor et al., 2003; Venables et al., 2005). In Arabidopsis (Arabidopsis thaliana), the polyunsaturated species, NAE 18:2 and NAE 18:3, together comprise >75% of the total NAE pool (Wang et al., 2006).

During seed germination and seedling establishment, NAE levels decrease dramatically, suggesting that the depletion of NAEs is an important requirement for normal seedling establishment (Chapman et al., 1999; Kilaru et al., 2012). Exogenous application of various NAE types, including NAE 12:0, NAE 18:2, and NAE 18:3, cause abnormal Arabidopsis seedling development (Teaster et al., 2007; Cotter et al., 2011; Keereetaweep et al., 2013, 2015; Blancaflor et al., 2014). Arabidopsis FATTY ACID AMIDE HYDROLASE
(FAAH) overexpressors, in which endogenous NAEs deplete more rapidly than in wild type, are insensitive to application of exogenous NAEs and display more robust seedling growth, suggesting that these small lipids act as negative growth regulators (Wang et al., 2006). Most intriguingly, exogenous application of NAE 18:3 not only results in reduced root elongation, but also inhibits cotyledon expansion and induces a degreening phenotype (Keereetaweep et al., 2013). The degreening process can be quantified by the loss of chlorophyll in seedlings and only occurs in cotyledons, not emerging true leaves. Seedlings are sensitive to NAE18:3 within a narrow window of development (3–5 d after sowing), and cotyledon bleaching is reversible when NAE18:3 levels are removed experimentally (Keereetaweep et al., 2013).

Two competing pathways, hydrolysis by FAAH and peroxidation by lipoxygenases, cooperate to contribute to the marked decline in total polyunsaturated NAE levels during seed germination and subsequent seedling establishment (Kilaru et al., 2012; Keereetaweep et al., 2013). Further experiments demonstrate that the hydro(pero)xides of NAE 18:2 and NAE 18:3, not the corresponding free fatty acid hydro(pero)xides, are the bioactive inhibitors of seedling development (Keereetaweep et al., 2013, 2015). The inhibitory action of 9-hydroxy-octadecadienoylethanolamide (9NAE-HOD) appears to elevate abscisic acid (ABA) synthesis in a feed-forward loop that then activates a growth inhibition program via an ABA receptor-mediated and ABSCISIC ACID INSENSITIVE3-dependent signaling pathway to reduce root elongation specifically (Keereetaweep et al., 2015). By contrast, it remains unclear as to the pathway(s) leading to cotyledon degreening and reduction of cotyledon expansion induced by oxylipin metabolites of NAE18:3, namely 9- and/or 13-hydroxy-octadecatryenoylethanolamide (9- and/or 13-NAE-HOT).

NAE 18:2-derived oxylipins (e.g. 9NAE-HOD) accumulate endogenously under unfavorable environmental conditions (drought simulation or salt treatment) to restrict, directly or indirectly, root elongation and seedling growth (Keereetaweep et al., 2015). By contrast, NAE-18:3–derived oxylipins are associated with inhibition of both root elongation and cotyledon expansion (Keereetaweep et al., 2013). NAE metabolism may be part of a fine-control mechanism that exists to help regulate the development of seedlings under adverse environmental conditions, partially through the ABA-mediated process of “secondary dormancy” discovered by Lopez-Molina et al. (2001, 2003).

In animals, polyunsaturated NAEs bind specific G-protein coupled receptors (GPCRs) and activate GPCR-catalyzed exchange of a guanine nucleotide bound to the heterotrimeric G-protein. Activation of G-protein signaling in turn regulates multiple downstream signaling pathways (Abadji et al., 1999; Bosier et al., 2010). Plants have a heterotrimeric G-protein complex but lack a significant complement of GPCRs because nucleotide exchange is spontaneous. Despite the lack of GPCRs, signaling in plants via activated heterotrimeric G-proteins is known to control numerous physiological processes including those involved in seed germination, root elongation, stress-mediated bleaching of cotyledons, and overall seedling establishment (Uran0 and Jones, 2014). Arabidopsis has one canonical Gα subunit (AtGPA1), one Gβ subunit (AGB1), and three Gγ subunits (AGG1, AGG2, and AGG3; Uran0 and Jones, 2014). In addition to AtGPA1, the Arabidopsis genome encodes three EXTRA-LARGE G-PROTEINs (XLG1, XLG2, and XLG3; Lee and Assmann, 1999; Ding et al., 2008; Uran0 et al., 2016). In addition, part of this G-protein complex is a 7-transmembrane (7TM) REGULATOR OF G-PROTEIN SIGNALING1 (AtRGS1; Chen et al., 2003) that serves to repress G-protein activation until its activity is inhibited, in part, by dissociation of AtRGS1 from the plasma membrane-localized G-protein complex (Uran0 et al., 2012; Fu et al., 2014). The AGB1/AGG dimer is the pivotal element of Arabidopsis G-protein signaling because it is required for the Gα subunit activity (Jones et al., 2011). Loss of AGB1 confers the most severe phenotypes (Ullah et al., 2003; Uran0 et al., 2013), whereas for genetic ablation of the canonical Gα subunit, AtGPA1, the severity is less than
for \textit{agb1} mutants. This is due in part to overlapping function of the atypical XLG proteins. Genetic ablation of both the canonical and atypical \( G\alpha \) proteins in \textit{Arabidopsis} confers a loss of \textit{AGB1} function (Urano et al., 2016), and similarly a single loss-of-function allele in \textit{agb1} completely disables the heterotrimeric G-protein pathway.

Here, we used a genetic approach to determine if the NAE-18:3 effects on seedling development reported previously (Keereetaweep et al., 2013) involve a defined signaling pathway. An informative suite of G-protein mutants was tested for visible NAE-18:3–elicited phenotypes. Whole transcriptome sequencing (RNA sequencing [RNA-seq]) with \( G\beta\) loss-of-function mutants (\textit{agb1}) revealed that a functional G-protein is required for NAE18:3-induced cotyledon degreening. Together, our results reveal a new feature of the NAE lipid signaling pathway in plants, one that requires components of G-protein signaling. While curiously reminiscent of how NAEs

\begin{figure}
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Arabidopsis G-protein mutants are tolerant to exogenous NAE-18:3 treatment. Four-d-old \textit{Arabidopsis} seedlings (Col-0, \textit{gpa1}, \textit{rgs1}, \textit{agb1}, \textit{agg1}; \textit{agg2}, \textit{agg3}, and \textit{xlg1}; \textit{xlg2}; \textit{xlg3}) were treated with 0.02\% dimethyl sulfoxide (DMSO; 0-\textmu M NAE 18:3) or 10-, 20-, 30-, or 40-\textmu M NAE 18:3 for 24 h. Images of treated seedlings were taken and chlorophyll was extracted using 80\% (\textit{v/v}) acetone and measured spectrophotometrically. A, Representative images of \textit{Arabidopsis} seedlings after 24-h treatment with DMSO or NAE 18:3. B, Total chlorophyll content of seedlings exposed to DMSO or NAE 18:3 treatment for 24 h. Data are means \pm SD of three biological replicates with 15 seedlings per replicate. Significant differences are indicated by lowercase letters above histogram bars (\( P < 0.05 \)).}
\end{figure}
RESULTS

G-Protein Mutants Are Tolerant to NAE-18:3–Induced Cotyledon Bleaching

When 4-d-old Arabidopsis seedlings were treated with NAE 18:3, cotyledons bleached and overall seedling growth was inhibited (Fig. 1; Keereetaweep et al., 2013). To determine if G-proteins were involved in the NAE-18:3 signaling pathway, a group of Arabidopsis G-protein mutants were treated with NAE 18:3 and cotyledon bleaching was assessed. The agb1, agg1; agg2; agg3 and xlg1; and xlg2; xlg3 mutant seedlings were relatively more tolerant to NAE-18:3 treatment than Col-0 (Fig. 1). Although overall seedling growth was reduced in tolerant mutants (Fig. 1A), the cotyledons remained green and this was quantified by measuring total chlorophyll content in treated seedlings. The chlorophyll content was normalized to fresh weight, and, consistent with the visible phenotypes, chlorophyll content was reduced more in Col-0 relative to agb1, agg1; agg2; agg3, and xlg1; xlg2; xlg3 mutants ($P < 0.01$).

By contrast, NAE 18:3 decreased chlorophyll content in rgs1, and gpai more than Col-0 (rgs1 $P < 0.05$; gpai $P < 0.01$; Fig. 1B). The opposite phenotypes of the single gpai mutant compared to the xlg triple mutant suggest that XLG proteins are the primary partners to the AGB1/AGG dimer. This result is also consistent with the visible phenotypes shown in Figure 1A. Collectively, these results suggest that G-protein subunits $G_b$, $G_y$, and XLG, but not Go or AtRGS1, are likely involved in NAE-18:3–induced degreening of Arabidopsis seed cotyledons.

AtRGS1 Internalizes in Response to NAE-18:3 Treatment

Previous studies have shown that G-protein signaling activation in plants is initiated with the endocytosis of AtRGS1 (Uran, et al., 2012; Fu et al., 2014; Tunc-Ozdemir et al., 2016). To determine the effect of NAE 18:3 on the internalization of RGS1, epidermal cells expressing AtRGS1–yellow fluorescent protein (YFP) were treated with NAE 18:3 and imaged using confocal microscopy. Treatment with $100 \mu M$ of NAE 18:3 showed increased levels of AtRGS1-YFP internalization relative to controls without NAE (Fig. 2A). A time-course analysis showed a gradual increase in the internalization of AtRGS1-YFP after treatment with either 40- or 100-$\mu M$ NAE 18:3 with a maximum value of 41% after 30 min of treatment (Fig. 2B). The level of internalization caused by NAE 18:3 is equivalent to what has been observed for other ligands, including the pathogen-associated molecular pattern flagellin peptide 22, a known activator of G-protein signaling (Fu et al., 2014). These results suggest that NAE 18:3 triggers AtRGS1-YFP internalization, resulting in activation of G-protein signaling.

RNA-Seq Reveals the AGB1-Dependent Transcriptome Changes Induced by 1-h and 3-h NAE-18:3 Treatment

RNA-seq was used to determine the early AGB1-dependent transcriptome changes induced by NAE18:3. Arabidopsis Col-0 and agb1 seedlings were treated with NAE 18:3 for 1 or 3 h and collected for Illumina-based, RNA-seq analysis. Three biological replicates (15 seedlings per replicate) from each genotype and treatment condition were used for RNA-seq. After 1 or 3 h of NAE-18:3 treatment, the expression of several genes was significantly altered in Col-0 and agb1 seedlings based upon the cutoff of false discovery rate (FDR) < 0.01 and log-log of fold change (logFC), $-1 \leq \text{logFC} \leq 1$ (Fig. 2). The complete list of differentially expressed genes (DEGs) with logFC and FDR is provided in Supplemental Table S1. AGB1-dependent genes were identified when Col-0 and agb1 DEGs were compared after 1 h or 3 h of NAE-18:3 treatment (Fig. 3B). After 1 h of NAE-18:3 treatment, transcript abundance of 214 genes increased and of 88 genes decreased in an AGB1-dependent manner (Fig. 3B). After 3 h of NAE-18:3 treatment, AGB1-dependent gene expression was more dramatically evident with transcript abundance elevating for 948 genes and declining for 1,192 genes (Fig. 3B).

Gene-ontology (GO) enrichment analysis identified a statistically significant number of genes that encode proteins involved in several biological processes and molecular functions in specific subcellular locations (Supplemental Figs. S1–S4). After 1 h of NAE-18:3 treatment, the significantly enriched GO terms were similar in Col-0 and agb1 seedlings (Supplemental Figs. S1, A and B). Further, while the majority of the biological processes that involved transcripts with increased abundance were similar in NAE-18:3–treated Col-0 and agb1 seedlings at 3 h (Supplemental Fig. S2A), some, including response to topologically incorrect protein (GO:0035966) and programmed cell death (GO:0012501), were specific to NAE-18:3–treated Col-0 (corresponding DEGs enriched in Col-0 but not in agb1 post treatment), revealing processes regulated by AGB1. Similarly, AGB1-dependent biological processes, including pigment biosynthetic process (GO:0046148), stomatal complex development (GO:0010374), flavonoid biosynthetic/metabolic process (GO:00009813/GO:0009812), ion transport (GO:0006811), and polysaccharide metabolic process (GO:0005976; Supplemental Fig. S2B) were observed when GO analysis was performed on transcripts with decreased abundance.
The molecular functions enriched in genes with increased transcript abundance as a result of NAE-18:3 treatment in both Col-0 and agb1 seedlings after 1 h included ubiquitin-protein/ubiquitin-like protein transferase activity (GO:0004842/GO:0019787), small-molecule binding (GO:0036094), and heat shock protein binding (GO:0031072; Supplemental Fig. S3A). In Col-0 seedlings, a significant number of transcripts with abundance reduced by NAE-18:3 treatment encoded proteins with specific functional activities including transmembrane receptor protein receptor kinase activity (GO:0019199), microtubule motor activity (GO:0003777), and hydrolase activities (GO:0016798 and GO:0004553; Supplemental Fig. S3B) and a significant number were part of the kinesin complex (GO:0005871) or located in the chloroplast (GO:0009507; Supplemental Fig. S4B). Because agb1 mutants were more tolerant to NAE-18:3 treatment, the AGB1-dependent DEGs and the cellular processes, functions, and locations of the proteins encoded by these genes likely contribute to the NAE-18:3–induced, AGB1-dependent process of cotyledon bleaching.

**Heterotrimeric G-Proteins and G-Protein Interactor-Encoding Genes Are Differentially Expressed in an AGB1-Dependent Manner**

The relative expression level and enrichment of genes that encode G-protein interactors were examined using the publicly available database Biological General Repository for Interaction Datasets (thebiogrid.org; Fig. 4; Oughtred et al., 2019). The complete list of DEGs that encode Arabidopsis G-protein interactors is included in Supplemental Table S2. After removing duplicates, 56 proteins were identified as Gα interactors, and, using the GeneOverlap program (Shen and Sinai, 2019) in R (R Core Team, 2018), we determined that there was an enrichment of genes that encode Gα-interacting proteins in the list of NAE-18:3 DEGs (19 genes; \( P \) value = 0.02; Fig. 4A). Of these 19 genes, the expression levels of the...
RNA-seq analysis showed NAE-18:3 treatment led to relatively more transcriptome changes in Col-0 seedlings relative to agb1 seedlings. NAE-18:3–treated Col-0 and agb1 seedlings were analyzed for DEGs (FDR < 0.01 and $-1 \leq \log_{2}FC \leq 1$) by comparing with DMSO-treated seedlings using differential expression analysis. A, The number of up- or downregulated genes in Col-0 or agb1 after 1 or 3 h of NAE-18:3 treatment. B, Venn diagram showing the number of genes specifically induced (upregulated) or repressed (downregulated) in Col-0 and agb1 after 1 and 3 h of NAE-18:3 treatment. The genes induced or repressed in
eight genes were significantly altered in an AGB1-dependent manner (Fig. 4A). The transcripts from the gene that encodes AtRGS1 (AT3G26090) increased in abundance in an AGB1-dependent manner. As briefly described above, AtRGS1 contains a 7TM domain similar in topology to GPCRs and maintains the Arabidopsis G-protein complex in an inactive state by acting as a GTPase-accelerating protein (Chen et al., 2003). VASCULAR PLANT ONE ZINC FINGER PROTEIN2 (VOZ2) transcripts increased in an AGB1-dependent manner. Transcripts of genes encoding two AtGPA1 interactors, SPX DOMAIN GENE1 and LEU RICH REPEAT PROTEIN1, decreased in abundance in an AGB1-dependent manner.

There also was a significant overlap between the genes that encode Gβ-interacting proteins and genes that were differentially expressed after 1 h and/or 3 h of NAE-18:3 treatment (31 genes; P value = 4.6 × 10⁻³; Fig. 4A). Of the 31 DEGs that encode Gβ interactors, 11 genes were altered in an AGB1-dependent manner. Three of these 11 genes were components of the Arabidopsis heterotrimeric G-protein complex, namely, AtGPA1, AGG1, and AGG2. VOZ2, previously described as an AtGPA1 interactor, was also part of the Gβ interactome. In addition, three of the Gβ-interacting proteins encoded by genes whose expressions are differentially modulated by NAE-18:3 treatment show differential phosphorylation in the gpa 1–4; agb 1–2; agg 1–1; agg 2–1 quadruple mutant (designated quad hereafter) when compared to wild type (Song et al., 2018).

The Biological General Repository for Interaction Datasets contained 149 nonredundant interactors of AtRGS1. There was a statistically significant overlap of genes that encode AtRGS1 protein interactors and genes that were differentially expressed after 1 h and/or 3 h of NAE-18:3 treatment (51 genes, P value = 1.1 × 10⁻⁴; Fig. 4B), and the change in expression of 18 of these interactor genes was AGB1-dependent. Transcripts from the gene that encodes SER/THR PROTEIN KINASE2 (S6K2) increased in abundance in an AGB1-dependent manner after 1 h of NAE-18:3 treatment. Transcripts from a GRAS-protein–encoding gene, PHYTOCHROME A SIGNALING TRANSDUCTION1, increased in abundance in an AGB1-dependent manner after 3 h of NAE-18:3 treatment. Transcripts from a senescence-associated gene, SENESCENCE1 (SEN1) also increased in abundance after NAE-18:3 treatment in an AGB1-dependent manner. Additionally, one of the AtRGS1-interacting proteins encoded by a NAE-18:3–induced gene is differentially phosphorylated in the quad mutant when compared to wild type (Song et al., 2018).

Genes encoding protein interactors of the three extra-large G-proteins, XLG1, XLG2, and XLG3, were differentially modulated as a result of 1 h and/or 3 h of NAE-18:3 treatment (Fig. 4C). There were seven DEGs that encode XLG1 interactors after 1 h and/or 3 h of NAE-18:3 treatment (seven genes; P value = 0.13). However, the expression of only two of these genes was significantly changed in an AGB1-dependent manner. In addition, transcripts from four genes that encode XLG2-interacting proteins increased after NAE-18:3 treatment. XLG3 protein interactor genes were also differentially expressed in an AGB1-dependent manner. Transcripts from three of these genes increased in abundance and transcripts from the other four decreased in abundance after 3 h of NAE-18:3 treatment (Fig. 4C). One of the NAE-18:3–repressed genes encoding a XLG3 protein interactor is differentially phosphorylated in the quad mutant when compared to wild type (Song et al., 2018).

In addition to modulating the expression of genes that encode G-protein interactors, NAE-18:3 treatment also led to a statistically significant increase in transcript abundance for several Arabidopsis G-protein subunits (Fig. 4D). Transcripts from the genes that encode the G-protein subunits, AtGPA1, AGB1, AGG1, AGG2, and AtRGS1, increased in abundance after 3 h of NAE-18:3 treatment. In addition, the expression of two extra-large G-protein genes, XLG2 and XLG3, also was higher after this treatment. The increase in expression of these G-protein subunit genes was AGB1-dependent, suggesting that a feed-forward transcriptional regulation of G-protein signaling is mediated by NAE18:3 through AGB1.

**Figure 3.** (Continued.)

Col-0 but not in agb1 mutants were considered AGB1-dependent DEGs, while genes induced or repressed in Col-0 and agb1 or just agb1 were considered AGB1-independent genes. C, Heat map showing DEGs in Col-0 and agb1 after 1 and/or 3 h of NAE-18:3 treatment.
sets, the expression of some of these genes can be activated by NAE 18:3 independent of AGB1 and/or ABA. Gene expression changes shown to be ABA-dependent, but independent of G-proteins, also were enriched in NAE-18:3 DEGs in Col-0 and agb1 seedlings (Fig. 5). Collectively, these results show that there may be overlap or interaction between the ABA- and NAE-18:3 signaling pathways that are AGB1-dependent or AGB1-independent. These results provide support for the conclusion that NAE 18:3 is a
A regulatory mode analysis was conducted to understand if our gene of interest overlapped with the Arabidopsis- and G-protein-signaling pathways. This analysis was performed using MapMan (planttfdb.cbi.pku.edu.cn/prediction.php; Jin et al., 2018). The Arabidopsis genome codes for the family of transcription factors that may act to repress these genes after NAE-18:3 treatment. The GLK family of transcription factors has also been shown to promote the expression of chlorophyll biosynthesis and photosynthesis-related genes (Rossini et al., 2001; Fitter et al., 2002; Yasumura et al., 2005; Waters et al., 2008, 2009; Powell et al., 2012). The Arabidopsis genome encodes two GLK transcription factors, GLK1 and GLK2. After 1 h of NAE-18:3 treatment, the expression of genes encoding these two transcription factors was not significantly different from that of the solvent control. However, after 3 h of NAE-18:3 treatment, transcripts of GLK1 decreased in abundance specifically in Col-0 and transcripts of GLK2 decreased in abundance in both Col-0 and agb1. The NAE-18:3-induced decrease in transcripts that encode chlorophyll biosynthesis enzymes and transcription factors that regulate this process offers a mechanistic explanation for the observed decrease in chlorophyll content in seedlings exposed to NAE 18:3.

Expression of Chlorophyll Biosynthesis Enzyme Genes Was Reduced in an AGB1-Dependent Manner

Given that the quantity of chlorophyll in Col-0 seedlings decreased as a result of NAE-18:3 treatment, we analyzed the expression level of genes that encode enzymes in the tetrapyrrole and chlorophyll biosynthesis pathways (Fig. 6A). Transcripts from seven genes that encode enzymes involved in four key steps of chlorophyll biosynthesis decreased in abundance in an AGB1-dependent manner after 3 h of NAE-18:3 treatment. Transcript abundance for genes encoding these two transcription factors was not altered in an AGB1-dependent manner. The Arabidopsis genome encodes two GLK transcription factors, GLK1 and GLK2. After 1 h of NAE-18:3 treatment, the expression of genes encoding these two transcription factors was not significantly different from that of the solvent control. However, after 3 h of NAE-18:3 treatment, transcripts of GLK1 decreased in abundance specifically in Col-0 and transcripts of GLK2 decreased in abundance in both Col-0 and agb1. The NAE-18:3-induced decrease in transcripts that encode chlorophyll biosynthesis enzymes and transcription factors that regulate this process offers a mechanistic explanation for the observed decrease in chlorophyll content in seedlings exposed to NAE 18:3.

Table S3. Regulon enrichment of AGB1 and ABA treatments. DEGs and groups of genes with specific enrichment returned 45 transcription factors that possess over-represented targets in this list of genes. This transcription factor enrichment returned 45 transcription factors that possess over-represented targets in this gene set (P value < 0.05; Fig. 6B). Two transcription factors, AGAMOUS-LIKE 20 and BABY BOOM, were predicted to target five out of the six chlorophyll biosynthesis genes in this query. Five transcription factors, GATA TRANSCRIPTION FACTOR 28 (GATA28), GATA TRANSCRIPTION FACTOR 1 (GATA1), CYCLING DOF FACTOR 5, OBFBINDING PROTEIN3, and BASIC PENTACYSTEINE6, were predicted to target three out of the six chlorophyll biosynthesis genes in this query. The complete list of transcription factors is included in Supplemental Table S4.

The decrease in expression of genes that encode chlorophyll biosynthesis enzymes may also be due to the repression of key members of the GATA NITRATE-INDUCIBLE CARBON-METABOLISM INVOLVED (GNC) and GOLDEN TWO-LIKE (GLK) transcription factor families. We examined the expression level of GNC and CYTOKININ-RESPONSE GATA FACTOR 1 (CGA1) after NAE-18:3 treatment in Col-0 and agb1 seedlings, but transcript levels for CGA1 and GNC were not altered in an AGB1-dependent manner.

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Transcripts of Senescence, Autophagy, and Chlorophyll Catabolic Enzyme Genes Increased with NAE-18:3 Treatment

When Arabidopsis seedlings were treated with NAE 18:3 for 24 h, cotyledons bleached in an AGB1-dependent manner, and the abundance of a molecule that activates distinct signaling pathways that overlap with the ABA- and G-protein signaling pathways. Results also show that NAE 18:3 initiates signaling events independent of these two pathways. The complete list of overlapping genes is included in Supplemental Table S3.

Figure 5. NAE-18:3 treatment led to an enrichment of genes that are induced or repressed by ABA treatment in Col-0 and agb1 seedlings. The numbers in each cell describe the level of enrichment and includes the P value and the number of overlapping genes in parentheses. The cells highlighted in green describe statistically significant (P < 0.05) overlap between NAE-18:3 DEGs and groups of genes with specific AGB1 and ABA regulatory modes (Pandey et al., 2010).
Figure 6. Genes encoding enzymes involved in chlorophyll biosynthesis were repressed in an AGB1-dependent manner after 3 h of NAE-18:3 treatment. A, Expression of genes that encode enzymes involved in four key steps of chlorophyll biosynthesis after 1 or 3 h of NAE-18:3 treatment. Magnesium-chelatase subunits, CHLH and CHL12; magnesium-protoporphyrin IX methyltransferase, CHLM; NADPH:protochlorophyllide oxidoreductase (PORA, PORB, and PORC). B, The 1,000-bp upstream regions location and gene expression level calculations of genes involved in chlorophyll biosynthesis after 1 or 3 h of NAE-18:3 treatment.
manner (Fig. 1). The cellular processes that facilitate this change are leaf senescence, autophagy, and chlorophyll catabolism (Hanaoka et al., 2002; Pruzinská et al., 2005). The expression levels of genes that encode proteins involved in promoting senescence (Li et al., 2012), autophagy, and the chlorophyll catabolic pathway (Pruzinská et al., 2005; Schelbert et al., 2009) were analyzed in an effort to identify transcriptional changes and molecular targets that may lead to or be involved in chlorophyll breakdown.

As a result of NAE-18:3 treatment, 30 genes that encode promoters of leaf senescence were differentially expressed (Fig. 7A). Eleven of these 30 genes were modulated in an AGB1-dependent manner. Transcripts from three genes that encode senescence-promoting transcription factors, i.e. NAC-LIKE, ACTIVATED BY AP3/PI (NAP), ORESARA1 (ORE1), and RELATED TO ABI3/VP1 (RAV1), were more abundant after 1 and/or 3 h of NAE-18:3 treatment. NAP and ORE1 are members of the NAC family of transcription factors (Guo and Gan, 2006; Kim et al., 2009). Transcripts from two genes involved in the ABA-promoted leaf senescence process (Lee et al., 2011; Zhang et al., 2012), i.e. RECEPTOR-LIKE PROTEIN KINASE1 and SENESCENCE ASSOCIATED GENE13, also increased in abundance after 3 h of NAE-18:3 treatment in an AGB1-dependent manner. Additionally, transcript levels of two genes that encode proteins involved in SA-promoted senescence (Morris et al., 2000; Xiao et al., 2010), i.e. NON-EXPRESSER OF PR GENES1 and ACYL COA-BINDING DOMAIN3, and two genes that encode enzymes involved in chlorophyll catabolism during senescence, i.e. PHEOPHYTIN PHEOPHORBIDE HYDROLASE and NON-YELLOWING1 OR STAY-GREEN1, also increased after NAE-18:3 treatment in an AGB1-dependent manner.

During senescence, the major cellular process that facilitates the degradation of chloroplasts is autophagy (Lin and Wittenbach, 1981; Wittenbach et al., 1982; Ishida et al., 2014). The transcript abundance of genes that encode multiple components of the Arabidopsis autophagy machinery was significantly increased by 3 h of NAE-18:3 treatment, and for several of these components this increase in expression was AGB1-dependent (Fig. 7A). Transcript abundance of AUTOPIHAGY5 (ATG5), AUTOPIHAGY12A (ATG12a), AUTOPIHAGY18A (ATG18a), UBIQUITIN-ASSOCIATED DOMAIN-CONTAINING PROTEIN28, and ATG8 INTERACTING PROTEIN1 increased after NAE-18:3 treatment in an AGB1-dependent manner.

In addition to genes involved in the cellular processes, senescence, and autophagy, transcript levels of genes that encode enzymes involved in five steps of the chlorophyll catabolic pathway increased after 3 h of NAE-18:3 treatment. Transcript abundance of two Arabidopsis CHLOROPHYLLASE (CLH) genes (CLH1 and CLH2) increased after NAE-18:3 treatment in Col-0 and that of CLH1 also increased in agb1. However, these two enzymes are not involved in the degradation of chlorophyll during senescence (Schenk et al., 2007). By contrast, transcript abundance of a gene that encodes the plastid-localized enzyme essential to chlorophyll breakdown during senescence, PPH (Schelbert et al., 2009), increased after 3 h of NAE-18:3 treatment in Col-0 and agb1 seedlings. Transcripts from PHEOPHORBIDE OXYGENASE and CYTOCHROME P450, FAMILY87, SUBFAMILY A, and POLYPEPTIDEDE9 (CYP89A9) genes also showed increased abundance after 3 h of NAE-18:3 treatment. By contrast to chlorophyll biosynthesis, transcript abundances of only half of the genes that encode enzymes in the chlorophyll catabolic pathway affected by NAE-18:3 treatment were changed in an AGB1-dependent manner.

### DISCUSSION

Application of NAE 18:3 results in reduced cotyledon expansion and a bleaching phenotype in a developmentally sensitive window of early seedling establishment (Keereetaweep et al., 2013). This bleaching process is reversible; cotyledons become green again when NAE 18:3 is removed or metabolized, and this phenomenon can be exploited to examine potential regulatory steps in seedling establishment through its manipulation. In this study, we showed that cotyledon degreening requires G-protein signaling components. To identify some of the earliest signaling components, specifically those requiring AGB1 in cotyledon greening, we employed global gene expression comparisons between agb1 and the corresponding wild-type background (Col-0). A comprehensive view was presented of the transcriptome remodeling that occurred at early time points (1 and 3 h) after NAE-18:3 treatment. We showed that Arabidopsis seedlings responded to NAE 18:3 rapidly at the transcriptional level; 912 and 5,728 Arabidopsis genes were significantly altered in their expression within 1- and 3-h incubation with NAE 18:3, respectively, suggesting that indeed the perturbation of normal seedling processes by elevation of this small molecule has the potential to influence major developmental signaling pathways. In addition, 33% and 37% of the genes significantly altered in expression after 1 h and 3 h of NAE-18:3 treatment, respectively, were altered in an AGB1-dependent manner. Based on these results, we concluded that G-protein signaling is an early target for NAE-18:3 signal transduction during seedling establishment.

We began this study by asking whether components of the eukaryotic NAE signaling pathway were...
evolutionarily conserved by screening a group of G-protein mutants (Fig. 1). This screen demonstrated that G\(_b\), G\(_g\), and XLG subunits were involved in the NAE-18:3 signaling pathway that leads to cotyledon bleaching. \(\text{agb1}\) phenotypes examined thus far can be attributed to one or more of the three identified G\(_g\) subunits (Urano and Jones, 2014) and AGG1, AGG2, and AGG3 represent the full complement of G\(_g\) subunits in Arabidopsis (Chakravorty et al., 2011; Thung et al., 2012), so our results are consistent with the notion that the G\(_b\) dimers function as one signaling element here and not as independent G\(_b\) or G\(_g\) subunits.

AtRGS1 is a 7TM protein harboring a RGS domain; the 7TM region is essential for localizing AtRGS1 to the plasma membrane, and the RGS domain binds to the Ga subunit to accelerate its GTPase activity (Chen et al., 2003). Interestingly, cotyledons of both \(\text{gpr1}\) and \(\text{rgs1}\) seedlings bleached more than wild type under NAE-18:3 treatment. In the absence of AtRGS1 or Ga, there is more free AGB1 at the plasma membrane, leading to an enhanced NAE-18:3-induced, G\(_b\)\(_g\) dimer-mediated chloroplast response. This result suggests that the NAE-18:3 signaling bypasses Ga and AtRGS1 and selectively transmits the signal via G\(_b\)\(_g\) dimers (Urano et al., 2012, 2013; Colaneri et al., 2014; Fu et al., 2014). This kind of signaling mechanism by G-proteins, in which only the G\(_b\) and G\(_g\) subunits are involved in the propagation of the signal, was named “classical route II” (Pandey et al., 2010) and was well supported in plant defense signaling. In Arabidopsis, loss of function of the G\(_b\) or G\(_g\) subunits leads to compromised resistance against a variety of fungal pathogens (Trusov et al., 2006, 2007; Delgado-Cerezo et al., 2012; Torres et al., 2013). The G\(_b\)\(_g\)-mediated signaling also contributes to defense against the model bacterial pathogen \textit{Pseudomonas syringae} by participating in programmed cell death and inducing reactive oxygen species (Ishikawa, 2009; Liu et al., 2013; Lorek et al., 2013; Torres et al., 2013). The classical route II was also manifested in CLAVATA3 signaling in Arabidopsis, in which G\(_b\) and G\(_g\) subunits, but not Ga, are involved (Ishida et al., 2016). Based on these studies, we hypothesized that NAE 18:3 initiates a series of signaling events utilizing some of the same components as pathogen perception and/or meristem developmental signaling pathways, and that the G\(_b\)\(_g\) dimers, but not the Ga subunit, transduced the signal into a common and conserved downstream signaling pathway.

**Figure 7.** NAE-18:3 treatment led to an increase in transcript abundance of genes encoding promoters of leaf senescence, autophagy, and enzymes in the chlorophyll catabolic pathway. A, Expression change of 30 genes encoding promoters of leaf senescence differentially modulated by NAE treatment for 1 and 3 h. B, The expression change of multiple components of the Arabidopsis autophagy machinery after 1 and 3 h of NAE-18:3 treatment. C, Expression change of genes that encode enzymes involved in four steps of the chlorophyll catabolism pathway after 1 and 3 h of NAE-18:3 treatment.
Given the small contribution from Ga and AtRGS1, the involvement of heterotrimeric G-proteins in NAE-18:3 signaling could be explained in two ways: either the Gβγ dimers act independently from Ga, or Ga is replaced by another protein for heterotrimer formation. The Arabidopsis genome contains at least three genes encoding Ga-like proteins that have been classified as XLGs (Lee and Assmann, 1999; Ding et al., 2008). Recent results suggest that plant XLG proteins originated from a canonical Ga subunit and retained prototypical interaction with Gβγ dimers (Maruta et al., 2015; Lou et al., 2019). Particularly, XLGs function as direct partners of Gβγ dimers and mediate the same defense pathways (Maruta et al., 2015; Urano et al., 2016). For example, after the recognition of flagellin peptide 22 by FLAGELLIN-SENSITIVE2, XLG2 is directly phosphorylated by the kinase BOTRYTIS-INDUCED KINASE 1 and as a result dissociates from the Gβγ dimer and mediates downstream signaling (Liang et al., 2017).

In this study, XLGs were also required for NAE-induced bleaching of seedling cotyledons. Thus, it is possible that XLGs cooperate with Gβγ dimers to function in the same way as described for defense signaling, which could be further investigated in future studies.

To comprehensively assess the role of G-proteins and their downstream targets during NAE-18:3 regulation of seedling development, we sequenced and compared the whole transcriptome of Col-0 and agb1 seedlings after treatment with DMSO (solvent control) or NAE-18:3 for 1 h or 3 h. Arabidopsis seedlings rapidly responded to NAE-18:3 treatment as shown by the number genes that were differentially modulated after 1 h and/or 3 h of NAE-18:3 treatment (Fig. 3). We compared the lists of genes differentially expressed in Col-0 and agb1 seedlings post treatment to identify genes transcriptionally regulated in an AG81-dependent manner (Fig. 3, B and C). NAE-18:3 increased the abundance of transcripts from multiple G-protein subunit genes in an AG81-dependent manner (Fig. 4D). The transcript abundances for genes encoding G-protein subunits, AtGPA1, AG81, AGG1, AGG2, AtRGS1, XLG1, and XLG3, were significantly higher after 3 h of NAE-18:3 treatment specifically in Col-0 seedlings. In addition, there were statistically significant enrichments of DEGs that encode protein interactors of Ga, Gβ, AtRGS1, and XLG1 after 1 h and/or 3 h of NAE-18:3 treatment and the expression of some of these genes was altered in an AG81-dependent manner (Fig. 4). Transcripts from VOZ2, a gene that encodes a transcription factor that interacts with Ga and Gβ (Klopfleisch et al., 2011), increased in abundance after NAE-18:3 treatment. VOZ2 interacts with PHYTOCHROME B (Yasui et al., 2012) and CONSTANS and promotes flowering (Kumar et al., 2018). In addition, VOZ2 acts as both a positive and negative regulator of biotic and abiotic stress responses and to modulate the Arabidopsis stress adaptation response (Nakai et al., 2013; Prasad et al., 2018). In addition, the transcript abundance of a gene encoding a component of the TARGET OF RAPAMYCIN (TOR) signaling pathway, S6K2, an interactor of AtRGS1 (Klopfleisch et al., 2011), also was increased by NAE-18:3 treatment. NAE-18:3 is induced during phosphate deprivation (Misson et al., 2005) and during cold and drought stress (Mizoguchi et al., 1995), and S6K2 is a direct target of TOR (Xiong and Sheen, 2012). TOR is a master regulator of the growth response to environmental stimuli (Deprost et al., 2007) and TOR silencing leads to chlorophyll breakdown (Deprost et al., 2007). Transcript abundance of another stress-associated gene, SEN1, was also increased by NAE-18:3 treatment. SEN1 is induced by phosphate starvation (Wu et al., 2003) and in Arabidopsis leaves during senescence (Oh et al., 1996). The transcriptional regulation of G-protein targets by NAE-18:3 suggests that molecular components of the light, TOR, and multiple stress response signaling pathways may act downstream of G-protein activation. Furthermore, G-proteins are important in the sensing of the carbon/nitrogen balance that ultimately controls chlorophyll content in Arabidopsis (Liang et al., 2017). Sensing of the carbon/nitrogen balance is important at the post-germination developmental arrest checkpoint when seedlings undergo a transition from heterotrophic growth to photoautotrophic growth (Aalto et al., 2012). During this critical period of development, seedlings monitor environmental conditions and nutrient availability and will arrest growth in the presence of adverse conditions (Lopez-Molina et al., 2001, 2003; Sato et al., 2009). It is during this post-germinative developmental window when seedlings are sensitive to exogenous treatment with NAE-18:2 and NAE-18:3 (Keereetaweep et al., 2013, 2015) and ABA (Lopez-Molina et al., 2001, 2003). The transcriptional regulation of genes that encode G-protein interactors involved in environmental and nutrient availability responses, the link between G-proteins and carbon/nitrogen sensing and the post-germinative window of sensitivity to NAEs, and adverse conditions, suggests that the link between NAE-18:3-induced cotyledon bleaching and G-protein signaling is through this critical post-germination developmental checkpoint. As previously suggested, there may be some overlap or interaction between the NAE-18:3 signaling pathway and ABA signaling pathway because ABA treatment leads to the accumulation of NAE-18:3–derived oxylipins in 4-d-old Arabidopsis seedlings (Keereetaweep et al., 2013). We compared previously identified AG81-dependent, ABA-induced transcriptome changes (Pandey et al., 2010) with NAE-18:3 transcriptome changes to identify DEGs that were NAE-18:3–induced and dependent or independent of AG81 and/or ABA (Fig. 5). Collectively, the enrichments of these distinct groups of genes supports the hypothesis that there are some overlapping and some unique NAE-18:3–induced changes when compared with ABA-dependent and AG81-dependent signaling pathways. However, because the ABA receptor mutant psy1; psy2; psy4; psy5; psy8 (Gonzalez-Guzman et al., 2012) is sensitive to NAE-18:3 treatment (Supplemental Fig. S5), the process
of NAE-18:3–induced cotyledon degreening does not require ABA perception. Although this result suggests that the NAE-18:3–induced process of cotyledon degreening does not involve ABA signaling pathways, we do not rule out that NAE 18:3 may interact with ABA in other cellular processes.

In addition to identifying signaling components that may link G-proteins to downstream signaling pathways, we also analyzed the expression level of genes that encode enzymes in the chlorophyll biosynthesis (Fig. 6) and chlorophyll catabolism (Fig. 7) pathways. Transcripts from six genes that encode chlorophyll biosynthesis enzymes decreased in abundance in an *AGB1*-dependent manner after 3 h of NAE-18:3 treatment (Fig. 6A). In addition, the transcript abundances of genes encoding transcription factors that modulate chlorophyll biosynthesis genes in Arabidopsis also were decreased by NAE-18:3 treatment (Fig. 6C). We also identified transcription factors that were predicted to bind to the 1,000-bp promoter regions upstream of the chlorophyll biosynthesis genes repressed by NAE-18:3 treatment (Fig. 6B). NAE-18:3 treatment also led to statistically significant changes in the expression of genes that encode proteins involved in promoting senescence (Fig. 7A), autophagy (Fig. 7B), and enzymes in the chlorophyll catabolic pathway (Fig. 7C). In addition, the transcript abundance of a gene coding for key regulator of chlorophyll degradation, SGR1 (Armstead et al., 2007; Park et al., 2007; Ren et al., 2007; Sakuraba et al., 2015), increased after 3 h of NAE-18:3 treatment in an *AGB1*-dependent manner (Fig. 7B). Collectively, these results indicated that NAE-18:3–induced cotyledon bleaching is a result of the repression of chlorophyll biosynthesis and an increase in chlorophyll catabolism like that associated with the processes of senescence and autophagy.

CONCLUSION

On the surface, the involvement of G-proteins in mediating NAE18:3-dependent processes in plants perhaps should not be that surprising because G-proteins are central to the mechanism for transducing NAE 20:4 signals in animal systems (Abadji et al., 1999; Bosier et al., 2010). However, NAE 20:4 is essentially absent from vascular plants (they do not synthesize arachidonic acid), and they do not have obvious homologs of the G-protein-coupled cannabinoid receptors that bind NAE 20:4. Hence, there are likely to be unique features for the perception and transduction of NAE signaling in plant systems, despite, as we now conclude, the apparent conservation of trimeric G-proteins as a cross-kingdom mechanism for NAE signal transduction. The extensive lists of downstream targets of Gβ and XLG proteins identified in the NAE-18:3 transcriptome offer straightforward strategies for future experiments to identify more detailed components and pathways involved in NAE-18:3 signaling.

In summary, our RNA-seq data provide new insights into the regulation of Arabidopsis seedling development by NAE 18:3 via G-proteins. Our results show that induction of stress response, autophagy, senescence, and chlorophyll catabolic genes and repression of chlorophyll biosynthesis genes are *AGB1*-dependent processes, leading to the visible phenotype of NAE-18:3–induced cotyledon degreening (Fig. 8). It should be noted that our current dataset was obtained from whole seedlings with 1-h and 3-h incubation with NAE 18:3 and represents an excellent framework on which to base future, detailed examination of tissue-specific and temporal factors that participate in seedling establishment. Perhaps in the future, a series of time-course experiments with specific ethanolamide oxylipin metabolites of NAE 18:3 and NAE 18:2 (e.g. 9NAE-HOD, 9NAE-HOT, and 13NAE-HOT) in dissected cotyledons and root tissues will provide dynamic and perhaps
more complete datasets that should help decipher the gene expression patterns and functional modules induced by these lipids in different tissues.

MATERIALS AND METHODS

Plant Growth and NAE-18.3 Treatment Assays

All mutants of Arabidopsis (Arabidopsis thaliana) were in the Col-0 background. The transfer DNA insertion mutant allele of AAG1, aag1-2, was described by Ullah et al. (2003). The transfer DNA insertion mutant allele of AGB1, agb1-2, was described by Ullah et al. (2003). The null allele of AGRSG1, rsg1-2, was described by Chen et al. (2003). The agg1-1;agg2-1;agg3-3 triple mutant was described by Thung et al. (2012). The xlg1-1; xlg2-1; xlg3-1 triple mutant was described by Ding et al. (2008). All of these mutants were kindly provided by the Jones lab at the University of NC.

Arabidopsis seeds were surface-sterilized using 70% (v/v) ethanol and 20% (v/v) bleach for 3 min each, rinsed three times with sterile, ultra-pure water, and then stratified in sterile, ultra-pure water for 3 d at 4°C in the dark for all experiments before sowing in liquid on half-strength Murashige and Skoog (MS) medium (Sigma-Aldrich) with 1% (v/v) Suc (Sigma-Aldrich). Germination and growth were maintained in controlled conditions with 16-h-light/8-h-dark cycle (100 μmol m−2 s−1) at 20°C to 22°C. For NAE-18.3 treatment assays, 4-d-old Arabidopsis Col-0 and G-protein mutant seedlings grown in liquid media were transferred to one-half strength MS with 1% (w/v) Suc media containing either NAE 18:3 (Cayman Chemical) or 0.02% (v/v) DMSO (Sigma-Aldrich). A z-stack series was acquired at 0.5-μm steps, and YFP excitation was at 514 nm and emission collection at 525 to 565 nm with a GaAsP detector. A z-stack series was acquired on the hypocotyl epidermis 2 to 4 mm below the cotyledons of seedlings treated with water or NAE 18.3. Statistical analysis was performed using the truncated Stranded mRNA prep kit (Illunina). Libraries were quantified by fluorometry, immobilized, and processed onto a flow cell, followed by 2 × 75-bp sequencing-by-synthesis on a Next-Seq 500 system (Illunina) with a high-output cassette (Illunina). Library construction and RNA-seq were performed by the University of North Texas Genomics Center (untnugenomicscenter.squarespace.com).

The quality of the RNA-seq data were initially analyzed using the program FastQC (https://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Reads were mapped onto the Arabidopsis genome (The Arabidopsis Information Resource) using the software STAR (v.2.6; Dobin et al., 2013). The software DESeq2 (v.1.22.1; Love et al., 2014) was used for differential expression analysis, and samples were grouped by genotype and compared by treatment. Principal component analysis was conducted to identify any potential outliers among the samples using the program pcacalculator (Marini and Binder, 2016). The GO term戈 filtering for determination of different DEGs was set at FDR < 0.01 and q < 1.0 log2(1.1). The RNA-seq data used in this study were deposited in the National Center for Biotechnology Information Gene Expression Omnibus repository and can be accessed using accession no. GSE140294.

Chlorophyll Extraction and Quantification

Chlorophyll content was quantified by spectrophotometry of samples prepared by 80% (v/v) acetone extraction. Five seedlings in each well were collected, weighed, and incubated at room temperature in the dark in a 1.5-mL tube with 1 mL of 80% (v/v) acetone solution for at least 24 h then clarified by centrifugation for 5 min at 15,000 rpm. The supernatant was collected, and absorbance was measured at wavelengths 645 and 663 nm (A645, and A663, Spectro Star Nano; BMG Labtech) using 96-well microplates. The amount of total chlorophyll was calculated following Arnon’s equation (Arnon, 1949) as follows: Total chlorophyll (μg mL−1) = 20.2 (A645) + 8.02 (A663). Chlorophyll content was normalized to fresh weight. NAE-18.3 treatment assays and chlorophyll extractions were replicated two times and included three biological replicates with five seedlings each. A one-way ANOVA was used to evaluate statistical differences between treatments of the same Arabidopsis genotype, and post hoc testing using Tukey’s honestly significant difference test was used to identify means that were significantly different.

G-Protein Activation Assays

AtrRSGI initialization was measured as described in Urano et al. (2012), Yu et al. (2014), and Tunc-Ozdemir et al. (2016). Briefly, Arabidopsis seeds expressing 35S:AgrRSGI-YPF (Urano et al., 2012) were sterilized and placed in 1-mL liquid one-quarter strength MS medium without sugar in 12-well plates and stratified at 5°C for 2 d, followed by 2 h light, then grown in darkness at RT for 3 d. For optimal results, the plates were kept in darkness but moved to the microscope room on the third day to acclimate. NAE 18.3 was applied to seedlings for 0, 15, 30, or 60 min before imaging. Image acquisition was conducted on a model no. LSM880 with a C-Apochromat 40×/1.2 NA water immersion objective (Zeiss). YFP excitation was at 514 nm and emission collection at 525 to 565 nm with a GaAsP detector. A z-stack series was acquired at 0.5-μm intervals between images. Image processing and RCSI internalization measurements were conducted with the Fiji distribution of the software ImageJ as described by Urano et al. (2012) with the following modification: internalized YFP fluorescence was measured and subtracted from total YFP fluorescent of individual cells, as opposed to total fluorescence of the hypocotyl image, as stated in Urano et al. (2012). Images were acquired on the hypocotyl epidermis 2 to 4 mm below the cotyledons of seedlings treated with water or NAE 18.3. Statistical analysis was performed using the software GraphPad Prism 7 (graphpad.com).

RNA Isolation, Complementary DNA Library Preparation, and Illumina Sequencing

Three biological replicates of each genotype (Col-0 or aag1), treatment condition (DMSO or 40-μM NAE 18:3), and treatment duration (1 h or 3 h) were collected and submitted for RNA-seq. Each replicate included 15 4-d-old Arabidopsis seedlings. Each Arabidopsis seedling sample was ground to a fine powder and used for RNA extraction. Briefly, 500 μL of Hot Borate buffer (Wu et al., 2002) and 175 μL of 20 mg mL−1 1 Proteinase K was added to each sample. After mixing, the sample and buffer solution were added to a lilac shredder column from a RNeasy Plant Mini Kit (Qiagen) and the manufacturer’s protocol from the RNeasy Plant Mini Kit was followed for the remainder of the extraction. RNA quantity and quality were determined using the Qubit Flurometer (Invitrogen) and the 2100 Bioanalyzer (Agilent). Poly(A) enrichment and library preparation were performed using the Truseq Stranded mRNA prep kit (Illunina). Libraries were quantified by fluorometry, immobilized, and processed onto a flow cell, followed by 2 × 75-bp sequencing-by-synthesis on a Next-Seq 500 system (Illunina) with a high-output cassette (Illunina). Library construction and RNA-seq were performed by the University of North Texas Genomics Center (untnugenomicscenter.squarespace.com).

Bioinformatics Analyses of DEGs

GO enrichment analyses were performed using g:Profiler (Reimand et al., 2016), a publicly available tool for the functional interpretation of gene lists, and the program REVIGO (Supek et al., 2011) was used to remove redundant GO terms employing the similarity value of 0.5. The ggplot2 package (Wickham, 2009) was used to generate bar plots in R (R Core Team, 2016) using the GO term T equation (log[2]T) ≥ 3. Venn diagrams were generated using the tool Gene List Venn Diagram (http://genevenn.sourceforge.net/). Heat maps were generated using conditional formatting in the software Microsoft Excel and the free FastQC (https://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) program. The relative expression level and enrichment of genes that encode G-protein interacting proteins was also examined using the publicly available database Biological General Repository for Interaction Datasets (thebiogrid.org; Oughtred et al., 2019) and the GeneOverlap program (Shen and Sinai, 2019) in R (R Core Team, 2018). The Plant Transcriptional Regulatory Map (planttransdb.cbi.pku.edu.cn/prediction.php; Jin et al., 2017) was used to identify transcription factors that have a statistically significant over-representation of targets in this list of chlorophyll biosynthesis genes.

Validation of RNA-Seq Data by Reverse Transcription Quantitative PCR

To validate RNA-seq data, the transcript levels of six genes were analyzed using reverse transcription quantitative RT-qPCR (RT-qPCR, Supplemental Table S5). The Invitrogen SuperScript VILLO cDNA Synthesis Kit (Thermo Fisher) was used to synthesize complementary DNA using 500 ng of total RNA. The complementary DNA was used as a template in a 20-μL qPCR reaction using the Applied Biosystems PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) following the manufacturer’s protocol. The primer sequences used are shown in Supplemental Table S5. Two reference genes, UBQ70 and ACT7, were used (Czechowski et al., 2005). The transcript abundance of each gene was quantified using RT-qPCR in an Applied Biosystems QuantStudio 3 Real-Time PCR System using the Comparative CΔCt (ΔΔCt) method (Thermo Fisher Scientific). The fold change of each gene was calculated in relation to the expression of the gene in the untreated samples using the 2−ΔΔCt method (Livak and Schmittgen, 2001; Supplemental Table S5).

In addition to validating these RNA-seq data using RT-qPCR, these results were compared to an independent RNA-seq experiment (Supplemental Table S6).
and RT-qPCR experiments (Supplemental Table S7) in which 4-d-old Arabidopsis seedlings were treated with 100 μM of NAE 18:3 for 1 h (National Center for Biotechnology Information Gene Expression Omnibus no. GSE151019). When these data sets were compared, several genes in the choryphyll catabolism (five genes), senescence (22 genes), and autophagy (seven genes) pathways showed statistically significant, gene expression changes in the same direction (i.e. up [blue]/down [red]; Supplemental Table S6). In addition, eight genes that were induced or repressed in 4-d-old Arabidopsis seedlings (Col-0) treated with 40 μM of NAE 18:3 for 1 or 3 h and 4-d-old Arabidopsis seedlings (Col-0) treated with 100 μM of NAE 18:3 for 1 h were confirmed using RT-qPCR (Supplemental Table S7).

Accession Numbers

The Arabidopsis Genome Initiative (www.arabidopsis.org) numbers for the genes mentioned in this article are as follows: ABI3 (AT3G24650), ACOYL COLA-BINDING DOMAIN3 (ACBP3; AT4G24220), AGPA1 (AT2G26300), AGB1 (AT4G34460), AGGI (AT3G63420), AGGJ (AT3G22942), AGGK (AT3G26035), AGAMOUS-LIKE 29 (AGL29; AT3G26560), AGL5 (AT3G17920), AGT12a (AT5G45210), AGT18a (AT5G67770), AGT2 INTERACTING PROTEIN1 (AT2; ATG54980), BABY BOOM (BBM; AT4G17430), BOTOYRS-INDUCED KGI-NA1E1 (BIK; AT2G36600), BASIC PENTACYSTEIN6 (BPC6; AT5G42520), CYCLING DOF FACTORS (CDF5; AT4G69570), CAIL (AT4G26150), CLH (AT1G3630), CHL2 (AT2G45980), CHL2 (AT1G19670), CONSTANS (CO; AT1G18940), CYTOSOLIC ASYMMETRIC PROTEIN (CYP19A9; AT1G30470), DIVINYL PROTOCROPHYLLOIDE-S VINYL REDUCTASE (DVR; AT3G18660), FAAH (AT4G64440), FLAGELIN-SENSITIVE2 (FLS2; AT5G6330), GATA2 (AT3G24050), GATA28 (AT1G16300), GATA29 (AT1G28570), GLK2 (AT4G41940), GNC (AT5G86800), LEUCINE RICH REPEAT PROTEIN (LRR1; AT1G16950), NAP (AT1G64900), NONEXPRESSER OF PR GENES (NPR1; AT1G44280), NON-YELLOWING OR STAY-GREEN1 (NYE1/SGR1; AT4G20920), OBI-BINDING PROTEIN3 (OBP3; AT3G53570), ORE1 (AT3G5610), PHEOCHROMOBIA A OXYGENASE (PAO; AT3G44880), PHYTOCHROME B (PHYB; AT1G1790), PHYTOCHROME A SIGNALING TRANSDUCTION1 (PPT1; AT4G18510), PORB (AT4G27440), PORC (AT1G03630), PP1 (AT1G38130), PYL1 (AT4G7930), PYL2 (AT2G26040), PYL4 (AT2G38310), PYL5 (AT3G05440), PYL8 (AT3G3160), PYR1 (AT1G18790), RELATED TO ABI3/VP1 (RAV1; AT1G31260), RECEPTOR-LIKE PROTEIN KINASE (RPK1; AT1G03630), S6K2 (AT3G08720), SENESCENCE ASOCIATED GENES13 (SAG113; AT5G92210), SEN1 (AT4G35770), SPX1 (AT3G20135), UBIQUITIN-ASSOCIATED DOMAIN-CONTAINING PROTEIN2B (UBAC2B; AT1G36110), VOZ2 (AT2G42400), XGL1 (AT2G23460), XGL2 (AT4G34390), and XGL3 (AT1G19930).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Significantly enriched GO terms after 3 h of NAE-18:3 treatment.

Supplemental Figure S2. Significantly enriched biological-process GO terms after 3 h of NAE-18:3 treatment.

Supplemental Figure S3. Significantly enriched molecular-function GO terms after 3 h of NAE-18:3 treatment.

Supplemental Figure S4. Significantly enriched cellular-component GO terms after 3 h of NAE-18:3 treatment.

Supplemental Figure S5. ABA receptor mutants (pyl1, pyl2, pyl5, and pyl8) are sensitive to exogenous NAE-18:3 treatment.

Supplemental Table S1. Genes induced or repressed in 4-d-old Arabidopsis seedlings, Col-0 or agb1, treated with 40 μM of NAE 18:3 for 1 or 3 h.

Supplemental Table S2. Genes differentially expressed in 4-d-old Arabidopsis seedlings, Col-0 or agb1, treated with 40 μM of NAE 18:3 for 1 or 3 h that encode protein interactors of G-protein subunits or interactors of G-protein subunits.

Supplemental Table S3. Genes differentially expressed in 4-d-old Arabidopsis seedlings, Col-0 or agb1, treated with 40 μM of NAE 18:3 for 1 or 3 h that are induced or repressed by ABA treatment in an ABI2-dependent or ABI2-independent manner (Pandey et al., 2010).

Supplemental Table S4. List of transcription factors that are predicted to bind 1,000-bp upstream region of six chlorophyll biosynthesis genes (ATIG03630, AT4G27440, AT4G25080, AT5G1630, AT5G18660, and AT5G45930).

Supplemental Table S5. RNA-seq validation of genes differentially expressed in 4-d-old Arabidopsis seedlings, Col-0 or agb1, treated with 40 μM of NAE 18:3 for 1 or 3 h using RT-qPCR.

Supplemental Table S6. Genes involved in cotyledon degreasing that are induced or repressed in 4-d-old Arabidopsis seedlings, Col-0 or agb1, treated with 40 μM of NAE 18:3 for 1 or 3 h and/or 4-d-old Arabidopsis seedlings, Col-0, treated with 100 μM of NAE 18:3 for 1 h.

Supplemental Table S7. Genes induced or repressed in 4-d-old Arabidopsis seedlings, Col-0, treated with 40 μM of NAE 18:3 for 1 or 3 h and 4-d-old Arabidopsis seedlings, Col-0, treated with 100 μM of NAE 18:3 for 1 h, which were confirmed by RT-qPCR.

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