Letter

The *genomes uncoupled* Mutants Are More Sensitive to Norflurazon Than Wild Type[1][OPEN]

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In plant biology, one of the most important discoveries of our time is that in addition to serving as the metabolic center of plant cells, plastids serve as sensors that emit signals. These signals serve as major regulators of the cell by affecting gene expression in the nucleus and by inducing the turnover of individual chloroplasts (Chan et al., 2016; Larkin, 2016). The idea that plastids emit signals was first proposed nearly 40 years ago and was based on the observation that the activities of enzymes associated with photosynthesis are reduced in the albino sectors of leaves from a variegated barley (*Hordeum vulgare*) mutant. Later, the expression of genes encoding the light-harvesting chlorophyll *a*/*b*-binding proteins of PSII (Lhcb) was found to also depend on chloroplast biogenesis. Numerous subsequent studies using mutant alleles and inhibitors that disrupt chloroplast function thoroughly characterized the effects of plastid dysfunction on nuclear gene expression—especially the expression of photosynthesis-associated nuclear genes (PhANGs), such as the *Lhcb* genes. These data provided evidence that chloroplasts emit signals that regulate the expression of nuclear genes (Oelmüller, 1989).

In 1993, Susek et al. isolated the first mutant alleles that disrupt plastid-to-nucleus signaling (Susek et al., 1993). Importantly, they repeatedly isolated mutant alleles of *GENOMES UNCOUPLED1* (*GUN1*). These data unequivocally demonstrated that indeed, when chloroplasts experience dysfunction, a specific signaling mechanism that depends on *GUN1* regulates gene expression in the nucleus. During the 25 years that followed the landmark paper by Susek et al. (1993), we learned that a number of distinct plastid-to-nucleus signaling mechanisms regulate nuclear gene expression and that these mechanisms affect numerous plastidic and extraplastidic processes in plants and algae, such as chloroplast and amyloplast biogenesis, seedling and leaf development, hormone responses, circadian rhythm, DNA replication, photosynthesis, and tolerance to both biotic and abiotic stress (Larkin, 2014, 2016; Chan et al., 2016).

After the first *gun* mutant screen by Susek et al. (1993), several additional *gun* mutant screens yielded many mutant alleles of genes that encode a chloroplastic pentatricopeptide repeat protein named GUN1, enzymes that contribute to tetrapyrrole metabolism, and a blue-light photoreceptor named cryptochrome1 (cry1). Although cloning these genes provided insight into some of the key proteins that contribute to this signaling and indicated that tetrapyrrole metabolism in the plastid can regulate gene expression in the nucleus (Fig. 1), many of the mechanistic details of this signaling remain unknown (Larkin, 2014, 2016).

Perhaps one of the reasons for these gaps in our knowledge is the confusion surrounding these mutants (Larkin, 2014, 2016). One of the most persistent misunderstandings is associated with the *gun* mutants that have deficiencies in tetrapyrrole metabolism (Fig. 1). Consistent with tetrapyrrole metabolism in the plastid affecting gene expression in the nucleus in Arabidopsis (*Arabidopsis thaliana*), heme and Mg-protoporphyrin IX serve as plastid signals that regulate gene expression in algae. In addition to regulating gene expression, Mg-protoporphyrin IX regulates DNA replication in *Cyanidioschyzon merolae* (Larkin, 2014). However, a large body of data conflicts with the idea that Mg-protoporphyrin IX or other Mg-porphyrins serve as plastid signals in plants (Larkin, 2016). Nonetheless, heme regulates diverse signaling mechanisms in bacteria, red algae, yeast, and animals (Terry and Smith, 2013; Larkin, 2016). Regarding *gun* mutants, loss-of-function alleles of genes that promote Mg-chelatase activity and gain-of-function alleles of a gene encoding an isozyme of ferrochelatase induce increases in PhANG expression when chloroplasts experience dysfunction. Although these data are consistent with increases in the biosynthesis of both heme and heme-derived bilins serving as plastid signals, the finding that PhANG

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Z.C. prepared the *gun1-101 hy5* double mutant; L.S. performed the experiments; R.M.L. and L.S. analyzed and interpreted the data; R.M.L. conceived of the study, supervised the experiments, and wrote the article.

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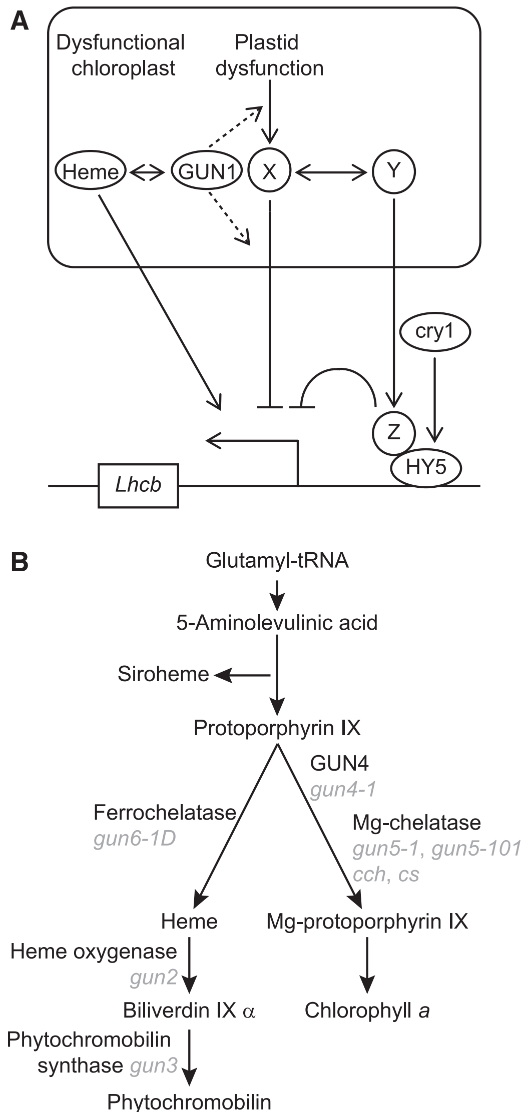

Figure 1. Plastid-to-nucleus signaling mechanisms defined by the *gun* mutants. A, Model for *GUN*-dependent plastid signaling. The *gun* mutants define three interacting plastid-to-nucleus signaling mechanisms that regulate the expression of *Lhcb* genes. When chloroplasts experience dysfunction, increases in heme biosynthesis up-regulate the expression of *Lhcb*. Additionally, *Lhcb* expression is downregulated by a currently unknown signal (X) that requires GUN1 for either its biosynthesis or transduction and a signaling mechanism that depends on an unknown signal (Y) that converts HY5 acting downstream of cry1 from a positive to a negative regulator of *Lhcb* expression, possibly by promoting the association of HY5 and a unknown factor (Z; Ruckle et al., 2007). Arrows indicate positive regulation. T bars indicate negative regulation. This conversion of light from a positive to a negative regulator appears unique to *Lhcb* genes (Ruckle et al., 2007). Plastid-to-nucleus signaling attenuates the light-induced expression of many other PhANGs (Ruckle et al., 2012) using a GUN1-dependent mechanism (Martín et al., 2016; data not shown). This distinct regulation of *Lhcb* expression by plastid signals may explain the enhanced sensitivity of *Lhcb* to plastid dysfunction that many laboratories have reported since the early days of plastid-to-nucleus signaling research (Oelmüller, 1989). B, Plastid-localized tetrapyrrole biosynthetic pathway of plants. The arrows indicate one or several enzyme-catalyzed reactions. Particular intermediates and enzymes are indicated with black text. Mutant alleles that alter the functions of particular enzymes are indicated with gray text. All of these mutant alleles are loss-of-function alleles except for *gun6-1D*, which is a gain-of-function allele (Larkin, 2016).

expression increases in a heme oxygenase mutant (i.e. *long hypocotyl1* [*hy1*/*gun2*]) with dysfunctional chloroplasts (Mochizuki et al., 2001) appears to rule out a role for increases in the biosynthesis of heme-derived bilins inducing PhANG expression by inducing the activity of phytochrome in the *gun* mutants. Nonetheless, in *Chlamydomonas* (*Chlamydomonas reinhardtii*), which lacks phytochrome, bilins serve as plastid signals that induce greening and the expression of PhANGs (Wittkopp et al., 2017). However, in the *gun* mutants with defects in tetrapyrrole metabolism, the only model consistent with all of the data is that increases in heme biosynthesis in the plastid serve as a signal that induces PhANG expression when chloroplasts experience dysfunction (Larkin, 2016).

To understand the confusion surrounding the *gun* mutants with deficiencies in tetrapyrrole metabolism, it is important to first understand the *gun* mutant screen. When Arabidopsis seeds are germinated in the light and in optimal conditions, the nonphotosynthetic proplastids—sometimes called eoplasts—in the cotyledons of mature embryos develop into chloroplasts, which are unique in that they accumulate chlorophyll (Pogson et al., 2015). In *gun* mutant screens, Arabidopsis seeds are germinated on a growth medium containing Suc and the herbicide norflurazon. The seedlings are grown for several days before their gene expression phenotypes are scored, using one or more reporter genes. During germination, norflurazon blocks chloroplast biogenesis by inhibiting the accumulation of carotenoids. Indeed, the plastids of norflurazon-treated seedlings resemble proplastids (Oelmüller, 1989; Susek et al., 1993). When norflurazon or a variety of distinct inhibitors or mutant alleles are used to block chloroplast biogenesis, PhANG expression is severely downregulated. Thus, PhANG expression is dependent on chloroplast biogenesis (Oelmüller, 1989). When chloroplast biogenesis is blocked, plastid gene expression is also downregulated (Woodson et al., 2013). The *gun* mutants express elevated levels of PhANGs when chloroplast biogenesis is blocked or when either chloroplast or etioplast function is somewhat attenuated, presumably because the *gun* alleles affect plastid-to-nucleus signaling mechanisms that regulate PhANG expression (Larkin, 2014, 2016; Chan et al., 2016).

A number of laboratories have promoted the idea that the *gun* alleles that disrupt tetrapyrrole metabolism do not affect a plastid-to-nucleus signaling mechanism but instead prevent norflurazon from completely blocking chloroplast biogenesis (Larkin, 2016). Thus, these *gun* mutants were proposed to express elevated levels of PhANGs, because PhANG expression is correlated with chloroplast biogenesis and because chloroplast biogenesis is proposed to reach a more advanced stage than in the wild type when seeds

from this group of *gun* mutants are germinated on a growth medium containing norflurazon. This criticism assumes that during germination on a growth medium containing norflurazon—when seedlings attempt to perform chloroplast biogenesis—chlorophyll or chlorophyll precursors accumulate and absorb light. In the absence of carotenoids, these electronically excited chlorophylls or chlorophyll precursors are proposed to transfer energy to molecular oxygen to yield singlet oxygen, which induces photo-oxidative damage that blocks chloroplast biogenesis (Oelmüller, 1989; Larkin, 2016).

Although still widely supported (Larkin, 2016), this model for the mechanism of norflurazon is largely based on data that was published more than 30 years ago (Oelmüller, 1989) that are subject to interpretation (Larkin, 2016). The main argument used to support this model is that low levels of chlorophyll accumulate in norflurazon-treated mustard (*Sinapis alba* L.) and barley (cv Carina) grown in dim light. When these norflurazon-treated plants were transferred to bright white light, both chlorophyll levels and PhANG expression were downregulated. Based on these data, it was suggested that singlet oxygen—derived from chlorophyll or its precursors—accumulated in these norflurazon-treated plants after they were transferred to bright white light and that this singlet oxygen damaged chloroplasts and downregulated PhANG expression (Oelmüller, 1989). However, more recently, when the accumulation of chlorophyll was completely blocked by germinating seedlings in dim light on a growth medium containing lincomycin—a light-independent inhibitor of plastid translation—transferring the lincomycin-treated albino seedlings from dim to bright light downregulated PhANG expression without inducing photo-oxidative stress (Ruckle et al., 2012). These data indicate that oxidative damage from singlet oxygen is not required and that interactions between light and plastid signaling are probably required to down-regulate PhANG expression when plants containing dysfunctional plastids are transferred from dim to bright light. Indeed, a large body of other data provides evidence for interactions between light and plastid signaling (Larkin, 2014; Martín et al., 2016). Consistent with these more recent findings, we now know that neither chlorophyll nor its precursors accumulate to detectable levels in seedlings that are grown on media containing norflurazon (Larkin, 2016). Furthermore, although norflurazon induces the high-level accumulation of singlet oxygen in green leaves (Kim and Apel, 2013), several different laboratories were unable to detect singlet oxygen in seedlings derived from seeds that were germinated on growth media containing norflurazon, using various techniques (Kim and Apel, 2013; Larkin, 2016; Page et al., 2017b). A large body of other data conflict with the idea that singlet oxygen derived from chlorophyll or chlorophyll precursors explains the gene expression phenotypes of these *gun* mutants. For example, there is no correlation between the chlorophyll-deficient phenotypes of the *gun* mutants and their gene expression phenotypes, screens for norflurazon-resistant mutants yielded mutants that are partially resistant to norflurazon but none of the previously isolated *gun* mutants, norflurazon appears to regulate different signaling mechanisms than singlet oxygen and other reactive oxygen species when seeds are germinated on a medium containing norflurazon, tetrapyrrole metabolism in the plastid was reported to affect gene expression in the nucleus in dark-grown Arabidopsis seedlings that were not treated with norflurazon, and tetrapyrrole metabolism was reported to regulate the expression of starch-associated nuclear genes in nonphotosynthetic cell-suspension cultures derived from tobacco (*Nicotiana tabacum* cv BY-2; Larkin, 2016). Moreover, during de-etiolation, *gun* mutants are more sensitive to increases in the fluence rate than wild type (Mochizuki et al., 1996; Ruckle et al., 2007).

An alternative model for the mechanism that norflurazon uses to block chloroplast biogenesis in germinating seedlings is that the inhibition of carotenoid biosynthesis by norflurazon leads to the down-regulation of chlorophyll biosynthesis because the biosynthesis of carotenoids and chlorophyll is coordinated. Down-regulating the biosynthesis of both of these pigments may block the biogenesis of the thylakoid membranes without inducing photo-oxidative stress (Kim and Apel, 2013). Nonetheless, the idea that norflurazon blocks chloroplast biogenesis by inducing photo-oxidative damage and, therefore, that the *gun* alleles that disrupt tetrapyrrole metabolism attenuate the production of singlet oxygen and are partially resistant to norflurazon stubbornly persists (Llamas et al., 2017; Page et al., 2017b; Hernández-Verdeja and Strand, 2018). Moreover, *gun* mutants and mutants that are partially resistant to norflurazon—*happy on norflurazon* (*hon*) mutants—are sometimes conflated (Saini et al., 2011; Page et al., 2017a). The retrograde signaling community appears to need an experiment that unambiguously resolves whether the *gun* mutants with defects in tetrapyrrole metabolism are partially resistant to norflurazon.

If the *gun* mutants with deficiencies in tetrapyrrole metabolism are less sensitive to norflurazon, this group of mutants should require higher concentrations of norflurazon to block chloroplast biogenesis than wild type. If this group of *gun* mutants affects plastid signaling, they may not require higher concentrations of norflurazon to block chloroplast biogenesis than wild type. To assay the sensitivity of these seedlings to norflurazon and the impact of norflurazon on chloroplast biogenesis, similar to other laboratories (Saini et al., 2011; Llamas et al., 2017), we chose to quantify the accumulation of chlorophyll. Inhibiting the accumulation of chlorophyll is one of the best-characterized effects of norflurazon on plants (Oelmüller, 1989). Additionally, the accumulation of chlorophyll is required for the biogenesis of the thylakoid membranes and photosynthesis—two unique and defining features of the chloroplast (Pogson et al., 2015). In the *gun* mutant screen, seeds are germinated on a medium containing

5 μM norflurazon (Susek et al., 1993), which is a higher concentration than is required to block chloroplast biogenesis in wild type. Indeed, 5 μM norflurazon blocks chloroplast biogenesis (i.e. prevents chlorophyll from accumulating) in wild type and in the *hon* mutants. However, although the *hon* mutants can accumulate chlorophyll when their seeds are germinated on a growth medium containing 50 nM norflurazon, chloroplast biogenesis is completely blocked when wild-type and particular *gun* mutant seeds are germinated on growth medium containing 50 nM norflurazon, as judged by the seedlings' ability to accumulate chlorophyll (Saini et al., 2011). Additionally, 25 nM norflurazon reduces the levels of chlorophyll of wild-type seedlings to approximately 50% of untreated wild-type seedlings (Llamas et al., 2017).

Because a *gun1* mutant and a *gun* mutant with defects in tetrapyrrole metabolism (i.e. *gun4-1*) did not appear resistant to norflurazon (Saini et al., 2011), we tested whether the *gun* alleles that disrupt tetrapyrrole metabolism might enhance a seedling's sensitivity to norflurazon. We germinated wild type, *gun5*, *gun5-101*, *cch*, *gun4-1*, and *cs* (Mochizuki et al., 2001; Larkin et al., 2003; Adhikari et al., 2011) on 1× Linsmaier and Skoog medium that contained 1% Suc and on the same medium containing 15 nM norflurazon. These mutants are deficient in a 140-kD porphyrin-binding subunit of Mg-chelatase named CHLH (*gun5*, *gun5-101*, *cch*), a 22-kD porphyrin-binding activator of Mg-chelatase named GUN4 (*gun4-1*), and a 40-kD subunit of Mg-chelatase that does not bind porphyrins (*cs*; Fig. 1). *gun5*, *gun5-101*, *cch*, and *gun4-1* are *gun* mutants (Mochizuki et al., 2001; Adhikari et al., 2011), but *cs* is not a *gun* mutant (Mochizuki et al., 2001). These mutants accumulated 25% to 65% of the chlorophyll that accumulates in wild type ($P < 0.0001$ to 0.003) when they were grown on a medium lacking norflurazon (Fig. 2), which is consistent with previous work (Mochizuki et al., 2001; Adhikari et al., 2011). We observed no significant difference in the levels of chlorophyll that accumulated in wild type when it was grown on a medium containing 15 nM norflurazon and when it was grown on the same medium lacking norflurazon ($P = 0.5$; Fig. 2). In contrast, when these mutants were grown on a medium containing 15 nM norflurazon, they accumulated only 14% to 29% of the chlorophyll that accumulated when the same genotype was grown on the same medium lacking norflurazon ($P < 0.0001$ to $P = 0.02$; Fig. 2). These data unequivocally indicate that *gun5*, *gun5-101*, *cch*, *gun4-1*, and *cs* are more sensitive to norflurazon than wild type and that these mutants are not resistant to norflurazon.

The idea that *gun* mutants are partially resistant to norflurazon is not limited to *gun* mutants that disrupt tetrapyrrole metabolism. *gun1* and *cry1* were also suggested to express elevated levels of PhANGs when chloroplast biogenesis is blocked because of reduced sensitivity to singlet oxygen derived from norflurazon (Saini et al., 2011; Terry and Smith, 2013). This idea conflicts with the finding that increases in light intensity inhibit chloroplast biogenesis in *gun1* and *cry1* more than in wild type (Mochizuki et al., 1996; Ruckle et al., 2007) and that *gun1-101* is more sensitive to diverse inhibitors of chloroplast biogenesis—including norflurazon—than wild type (Ruckle et al., 2012; Llamas et al., 2017). However, we do not know whether the *gun* mutants that disrupt light signaling (i.e. *cry1* and *hy5*) are more sensitive to norflurazon than wild type. We used the same conditions to grow wild type, *gun1-101*, *cry1*, and *hy5* as we used to grow the *gun* mutants that disrupt tetrapyrrole metabolism. We also analyzed the

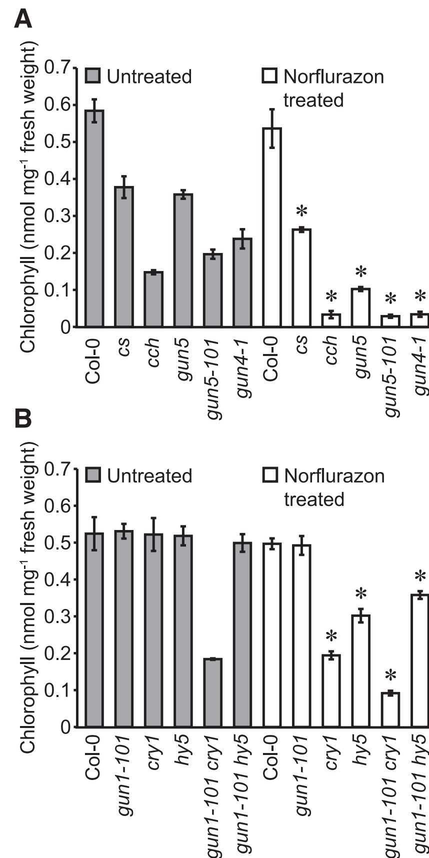

Figure 2. Chlorophyll levels in untreated and norflurazon-treated seedlings. A, Chlorophyll levels in untreated and norflurazon-treated mutants with deficiencies in tetrapyrrole metabolism. Seedlings were grown in continuous white light with a fluence rate of 80 μmol m^{-2} s^{-1} at 21°C for 4 d on 1× Linsmaier and Skoog medium containing 1% Suc (gray bars) and on the same medium containing 15 nM norflurazon (white bars). We extracted chlorophyll from 4-d-old seedlings using *N*, *N*′-dimethylformaide and quantified the levels of chlorophyll as described previously (Porra et al., 1989). We analyzed four biological replicates for wild type (Col-0) and each mutant. Error bars indicate 95% confidence intervals. * indicates a statistically significant difference relative to the same genotype grown on the same medium lacking norflurazon. Student's *t* test ($P < 0.001$ to 0.01). B, Chlorophyll levels in untreated and norflurazon-treated *gun*, *cry1*, and *hy5* mutants. The seedlings were grown and analyzed as described in A. * indicates statistically significant difference relative to the same genotype grown on the same medium lacking norflurazon calculated with a Student's *t* test ($P < 0.0001$ to 0.002).

gun1-101 cry1 and *gun1-101 hy5* double mutants because *Lhcb* expression is synergistically upregulated in these mutants when chloroplasts experience dysfunction (Ruckle et al., 2007). We found no significant difference in the levels of chlorophyll that accumulated in wild type and *gun1-101* when they were grown on a medium containing 15 nM norflurazon and when they were grown on the same medium lacking norflurazon ($P = 0.6$ and 0.3, respectively; Fig. 2), which is consistent with previous work (Llamas et al., 2017). However, we found that when *cry1*, *hy5*, *gun1-101 cry1*, and *gun1-101 hy5* were grown on a medium containing 15 nM norflurazon, they accumulated only 37% to 72% of the chlorophyll that accumulated when the same genotype was grown on a medium lacking norflurazon ($P < 0.0001$ to 0.002; Fig. 2).

To further characterize the response of these three groups of *gun* mutants to 15 nM norflurazon, we quantified the expression of genes that are associated with photosynthesis in wild type, *cch*, *gun4-1*, *gun1-101 cry1*, and *gun1-101 hy5*. We found that *Lhcb1.2* and *GOLDEN2-LIKE1* (*GLK1*) were expressed at similar levels in these mutants and wild type, regardless of whether they were treated with 15 nM norflurazon. However, *GLK1* was expressed at 3- and 8-fold lower levels in norflurazon-treated *gun4-1* and *cch* relative to untreated wild type ($P = 0.02$ and 0.03), respectively. Thus, for these mutants, quantifying the accumulation of chlorophyll provided a more sensitive chloroplast biogenesis assay than quantifying the expression of *Lhcb1.2* and *GLK1*. To test whether these *gun* mutants and wild type might accumulate different levels of reactive oxygen species, such as singlet oxygen, we quantified the expression of one general oxidative stress-responsive gene (*ZINC FINGER PROTEIN12*), two singlet oxygen-responsive genes (*WRKY33* and *BON ASSOCIATION PROTEIN1*), and one hydrogen peroxide-responsive gene (*FERRETIN1*). We attempted to quantify

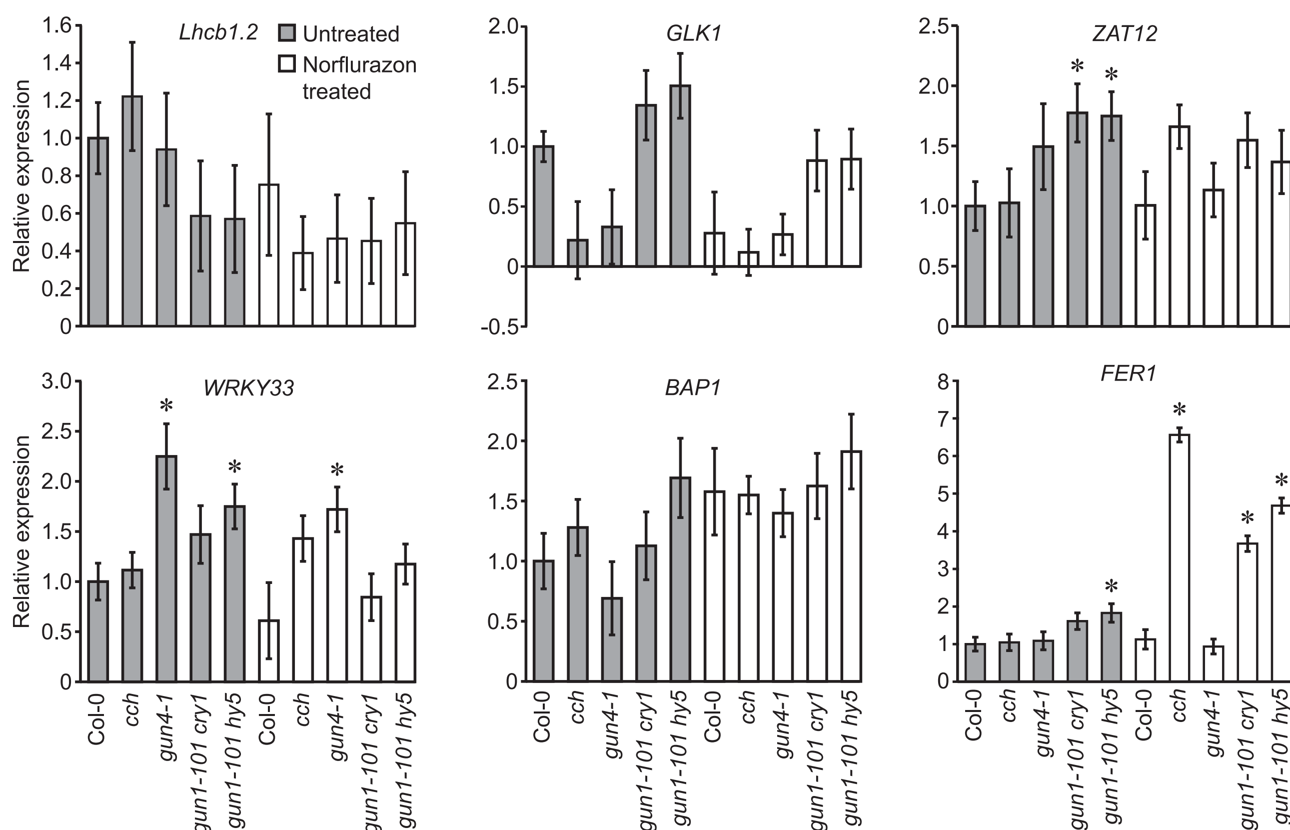

Figure 3. Expression of photosynthesis and oxidative stress-related genes in *gun* mutants. Seedlings were grown on a growth medium containing 15 nM norflurazon or the same growth medium without norflurazon as described in Figure 2. Gene expression was quantified using quantitative reverse transcription PCR and the comparative C_T method (Schmittgen and Livak, 2008). Expression is reported relative to wild type grown on a medium lacking norflurazon. We used previously reported primer pairs for *Lhcb1.2*, *GLK1* (Kakizaki et al., 2009), *ZINC FINGER PROTEIN12* (*ZAT12*; Doherty et al., 2009), *WRKY33* (Liu et al., 2017), *BON ASSOCIATION PROTEIN1* (*BAP1*; Baruah et al., 2009), *FERRETIN1* (*FER1*; Kim and Apel, 2013), and *ACTIN2* (Tang et al., 2010). Expression was normalized to the expression of *ACTIN2*. Four biological replicates were analyzed for each genotype in each condition. Error bars indicate SD. * indicates a statistically significant difference calculated with a Student's *t* test relative to wild type (Col-0) grown on the same growth medium ($P < 0.0001$ to 0.05).

the expression of a third singlet oxygen-responsive gene that encodes a member of the ATPases associated with diverse cellular activities (AAA) protein family commonly referred to as *AAA-ATPase* using primer pairs from two different papers (Baruah et al., 2009; Kim and Apel, 2013). However, we could not reliably quantify the expression of *AAA-ATPase* because its levels of expression were too low (L.S., unpublished data). The expression levels of the other genes provide evidence that these *gun* mutants experience similar or higher levels of oxidative stress relative to wild type. Most of the changes in oxidative-stress-associated gene expression were 1.5- to 2.8-fold increases (Fig. 3). We observed the greatest differences in the expression of *FER1*, which was expressed at 3.3- to 5.8-fold higher levels in norflurazon-treated *cch*, *gun1-101 cry1*, and *gun1-101 hy5* relative to norflurazon-treated wild type (Fig. 3). Nonetheless, *FER1* was not expressed at significantly different levels in *gun4-1* and wild type (Fig. 3). These data conflict with the idea that these *gun* mutants express elevated levels of *Lhcb* genes and other PhANGs when chloroplast biogenesis is blocked with norflurazon because they accumulate reduced levels of singlet oxygen and experience lower levels of oxidative stress relative to the wild type.

In summary, our findings indicate that the *gun* mutants with deficiencies in tetrapyrrole metabolism and the *gun* mutants with deficiencies in light signaling are more sensitive to norflurazon than wild type. Importantly, these data and recently published work with *gun1-101* (Llamas et al., 2017) unequivocally rule out the possibility that any of the *gun* mutants express elevated levels of PhANGs relative to wild type because they are partially resistant to norflurazon. The most parsimonious interpretation of these data is that each of these *gun* mutants expresses elevated levels of PhANGs when chloroplasts experience dysfunction because of abnormalities in plastid signaling mechanisms that regulate the expression of nuclear genes, such as PhANGs. Our finding that *gun* mutants are more sensitive to an inhibitor of chloroplast biogenesis than wild type also provides evidence that the *GUN* genes promote chloroplast biogenesis, which is consistent with previous de-etiolation experiments (Mochizuki et al., 1996; Ruckle et al., 2007) and the expression pattern of the GUN1 protein (Wu et al., 2018). Additionally, our data indicate that norflurazon does not block the chloroplast biogenesis that occurs during germination by inducing transient, localized, or sustained increases in the levels of singlet oxygen. Our data are consistent with norflurazon blocking the chloroplast biogenesis that occurs during germination by inhibiting carotenoid biosynthesis and indirectly down-regulating chlorophyll biosynthesis, which blocks the biogenesis of the thylakoid membranes without inducing photo-oxidative stress. Our hope is that unequivocally resolving this misunderstanding will facilitate research on the plastid-signaling mechanisms defined by the *gun* mutants.

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