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RESEARCH PAPER

Plastid signalling under multiple conditions is accompanied by a common defect in RNA editing in plastids

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Abstract

Retrograde signalling from the plastid to the nucleus, also known as plastid signalling, plays a key role in coordinating nuclear gene expression with the functional state of plastids. Inhibitors that cause plastid dysfunction have been suggested to generate specific plastid signals related to their modes of action. However, the molecules involved in plastid signalling remain to be identified. Genetic studies indicate that the plastid-localized pentatricopeptide repeat protein GUN1 mediates signalling under several plastid signalling-related conditions. To elucidate further the nature of plastid signals, investigations were carried out to determine whether different plastid signal-inducing treatments had similar effects on plastids and on nuclear gene expression. It is demonstrated that norflurazon and lincomycin treatments and the *plastid protein import2-2* (*ppi2-2*) mutation, which causes a defect in plastid protein import, all resulted in similar changes at the gene expression level. Furthermore, it was observed that these three treatments resulted in defective RNA editing in plastids. This defect in RNA editing was not a secondary effect of down-regulation of pentatricopeptide repeat protein gene expression in the nucleus. The results indicate that these three treatments, which are known to induce plastid signals, affect RNA editing in plastids, suggesting an unprecedented link between plastid signalling and RNA editing.

Key words: Gene expression, lincomycin, norflurazon, plastid protein import, plastid signal, RNA editing.

Introduction

Plastids originate from cyanobacteria that were incorporated into the eukaryotic cell (Dyall *et al.*, 2004). During evolution, most genes that were originally encoded in the cyanobacterial genome were transferred to the nuclear genome (Kleine *et al.*, 2009*a*). Therefore, plastid biogenesis and function rely on the expression of nuclear genes and the import of their products into plastids (Inaba and Schnell, 2008; Jarvis, 2008; Li and Chiu, 2010). Gene transfer from the cyanobacterial ancestor to the host nuclear genome has inevitably created problems. For example, the genetic information required for the assembly of protein complexes, such as the photosystem complexes, is now spread over two separate genomes. Therefore, the control of gene expression in the separate genomes needs to be coordinated for plant cells to function correctly (Beck, 2005; Woodson and Chory, 2008). A regulated input of nuclear-encoded plastid proteins is a prerequisite for plastid biogenesis, and the expression of nuclear genes encoding plastid proteins must be tightly coordinated with the functional or metabolic state of the plastids (Kessler and Schnell, 2009; Inaba, 2010). For these reasons, retrograde signals from plastids to the nucleus, referred to as plastid signals, have been proposed to play key roles in coordinating nuclear gene expression and regulating biochemical processes in plastids (Nott *et al.*, 2006; Pogson *et al.*, 2008).

Plastid signals have been studied by analysing the photosynthesis-related nuclear gene expression in response

to plastid damage. The rationale for this is that plastids generate signals when experiencing dysfunction (Pfannschmidt, 2010). Several treatments have been suggested to induce plastids to generate plastid signals. For instance, treatment of plants with the herbicide norflurazon (NF) generates plastid signals, and it appears that mutants defective in the tetrapyrrole biosynthesis pathway fail to down-regulate the expression of photosynthesis-related genes in response to NF-associated plastid signals, although the actual signalling molecule remains obscure (Strand *et al.*, 2003; Mochizuki *et al.*, 2008; Moulin *et al.*, 2008). Likewise, inhibition of plastid protein synthesis by lincomycin (Lin) or other antibiotics generates a plastid signal that suppresses the expression of photosynthesis-related genes in the nucleus (Oelmuller *et al.*, 1986; Mulo *et al.*, 2003). In previous work, it was shown that a defect in plastid protein import resulted in the generation of plastid signals and the subsequent down-regulation of expression of nuclear genes encoding plastid proteins (Kakizaki *et al.*, 2009). Previously published data point to a role for the plastid-localized pentatricopeptide repeat (PPR) protein GUN1 (Koussevitzky *et al.*, 2007; Kakizaki *et al.*, 2009) in relaying this signal to the nucleus. Independent of these pathways, EXECUTER proteins have been known to mediate signalling to the nucleus by plastid-derived singlet oxygen, which induces cell death (Wagner *et al.*, 2004; Lee *et al.*, 2007).

Plastid signals are often classified into several major groups based on their origins (Inaba, 2010). However, this classification depends on the experimental conditions and the strategies chosen for each study (Kleine *et al.*, 2009*b*; Pfannschmidt, 2010), and it remains unclear whether each treatment actually generates distinct signalling molecules. For example, the plastid signal induced by NF treatment was proposed to be the chlorophyll precursor magnesium protoporphyrin IX (Mg-ProtoIX) (Strand *et al.*, 2003). However, according to more recent studies, it is more likely that sensing of metabolite fluxes by tetrapyrrole-synthesizing enzymes, rather than the accumulation of Mg-ProtoIX, plays a central role in this signalling pathway (Mochizuki *et al.*, 2008; Moulin *et al.*, 2008). Despite numerous efforts, the plastid signals induced by each treatment have not yet been identified. On the other hand, the involvement of GUN1 in relaying signals both after NF and Lin treatment and in the context of a protein import defect raises another possibility (Koussevitzky *et al.*, 2007; Kakizaki *et al.*, 2009): each of these treatments may affect a similar, if not identical, plastid signalling process, thereby using GUN1 as a common signalling component. Indeed, *Arabidopsis* seedlings treated with NF or Lin, as well as seedlings from the *plastid protein import2-2* (*ppi2-2*) mutant, which lacks the atToc159 protein import receptor, all showed a severe albino phenotype (Fig. 1A). However, the possible nature of a plastid signal that is common to, or similar in, all of these treatments remains to be characterized.

Here it is demonstrated that RNA editing in plastids is affected by three different plastid signal-inducing treatments. The results therefore suggest an unprecedented link between plastid signalling and RNA editing.

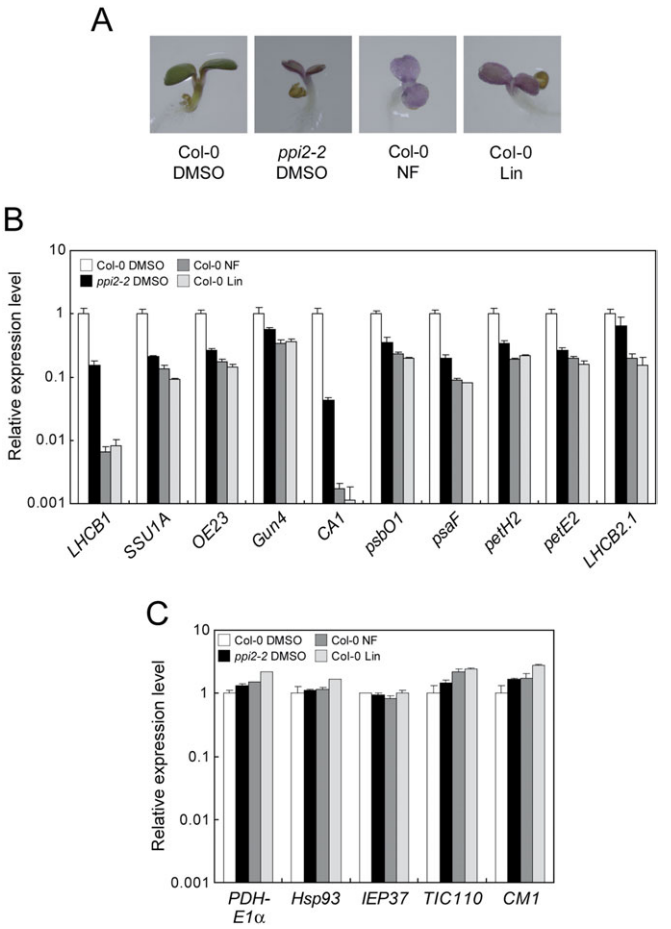

Fig. 1. Effects on *Arabidopsis* seedlings of the *ppi2-2* mutation and of treatment with norflurazon (NF) or lincomycin (Lin). (A) Phenotype of wild-type and *ppi2-2* seedlings grown on either 1 μM NF, 500 μM Lin, or DMSO for 5 d. (B and C) Expression profiles of genes encoding photosynthesis-related (B) and non-photosynthetic (C) proteins in *ppi2-2* mutant and wild-type plants treated with either 1 μM NF or 500 μM Lin. Wild-type plants treated with DMSO were analysed as a control. Plants were grown under continuous light (~125 μmol m^{-2} s^{-1}) for 5 d. Transcript levels were analysed by real-time PCR and normalized to the levels of *ACTIN2*. Each bar is plotted on a log scale and represents the mean of at least three independent samples. Error bars represent the standard deviations of the mean. AGI codes are listed in Supplementary Table S1 at *JXB* online.

Materials and methods

Plant material and growth conditions

All experiments were performed using *Arabidopsis thaliana* accession Columbia (Col-0). The *ppi2-2*, *gun1-101*, and *gun1-101 ppi2-2* mutant lines have been described previously (Kakizaki *et al.*, 2009). Plants were grown on 0.5% agar medium containing 1% sucrose and 0.5× MS salts at pH 5.8. To synchronize germination, all seeds were kept at 4 °C for 2 d after sowing. Plants were grown on agar medium containing 1 μM NF, 500 μM Lin, or dimethylsulphoxide (DMSO) under continuous light (~125 μmol m^{-2} s^{-1}) for 5 d. In experiments involving the *gun1-101 ppi2-2* double mutant, the double mutant seedlings were obtained using embryo rescue experiments as previously described (Kakizaki *et al.*, 2009).

RNA isolation and real-time PCR analysis

Total RNA was extracted from leaf tissue of wild-type and mutant plants using an RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized using the PrimeScript™ RT reagent kit (TaKaRa) with random hexamer and oligo d(T) primers. Real-time PCR was performed in a Thermal Cycler Dice™ Real-Time System (TaKaRa) using SYBR Premix Ex Taq (TaKaRa). The primers used for real-time PCR are listed in Supplementary Table S1 available at *JXB* online. The transcript level of each gene was normalized to that of *ACTIN2*.

Analysis of RNA editing

PCRs were performed using cDNA synthesized from *ppi2-2*, *gun1-101*, and *gun1-101 ppi2-2* mutant plants and wild-type plants treated with NF or Lin. The primers used for amplification of each PCR product are summarized in Supplementary Table S2 at *JXB* online. As a negative control, the same PCR conditions were used on RNA samples without the cDNA synthesis step. PCR products were sequenced directly to determine editing efficiencies. For quantitative analysis of RNA editing, amplified cDNA fragments were cloned into the *Eco*RV site of plasmid pZErO-2 (Invitrogen). Cloned fragments were subsequently amplified directly from the *Escherichia coli* colonies and the PCR products were sequenced. At least 99 independent clones were sequenced for the analysis of each gene.

Results

Effects of NF and Lin treatments and the ppi2-2 mutation on the expression of nuclear genes encoding plastid proteins

Plastid signals can be generated under several different conditions, including NF treatment, Lin treatment, and a defect in plastid protein import. Notably, these three different treatments all resulted in a severe albino phenotype, indicative of severely aberrant chloroplasts (Fig. 1A). Notwithstanding the fact that many conditions might cause this effect, it was decided to investigate whether the treated plants were similar at the molecular level. First the changes in expression of nuclear-encoded, photosynthesis-related genes and of genes that are unrelated to photosynthesis were examined. The *ppi2-2* mutant was chosen to investigate the effects of a chloroplast protein import defect on nuclear gene expression, as this mutant was previously used to analyse the link between plastid protein import and plastid-to-nucleus retrograde signalling (Kakizaki *et al.*, 2009). Total RNA was extracted from 5-day-old wild-type *Arabidopsis* treated with NF or Lin and from 5-day-old *ppi2-2* mutant seedlings. Real-time PCR analysis revealed that all photosynthesis-related genes examined were down-regulated in these plants (Fig. 1B). The effect was stronger in plants treated with NF or Lin than in the *ppi2-2* mutants, but the patterns were very similar in each of the three groups. Next the effect of each of the treatments on the expression of nuclear genes encoding proteins unrelated to photosynthesis was examined. As shown in Fig. 1C, most genes, regardless of their functional classification, showed similar expression patterns in the NF- and Lin-treated plants and in the *ppi2-2* plants. Overall, these results suggest that the transcriptional responses to NF or Lin treatment of nuclear genes encoding plastid proteins are similar to the transcriptional changes caused by the *ppi2-2* mutation.

Transcriptional responses of genes encoding nuclear transcription factors

Experiments were next carried out to examine whether plastid signals induced by Lin treatment, NF treatment, or the *ppi2-2* mutation utilize similar signalling pathways. According to the current model, chloroplast plastid signalling pathways activated by NF, Lin, and defects in protein import, and possibly others as well, converge at the GUN1 protein (Koussevitzky *et al.*, 2007; Kakizaki *et al.*, 2009; Inaba, 2010). Downstream of GUN1, however, the signalling pathways seem to diverge and utilize different combinations of transcription factors. Signals generated by NF treatment utilize both the GLK1 and ABI4 transcription factors (Koussevitzky *et al.*, 2007; Kakizaki *et al.*, 2009). In contrast, signals generated by a defect in plastid protein import use the GLK1 pathway but not the ABI4 pathway (Kakizaki *et al.*, 2009). Therefore, whether other transcription factors are also differentially regulated by the different treatments was determined. First 16 transcription factor genes that are down-regulated in rosette leaves of *ppi2-1* plants, as shown by SuperSAGE analysis (Kakizaki *et al.*, 2009), were examined. Previously, *GLK1* was also identified as a component of the plastid signalling pathway based on this SuperSAGE analysis. Real-time PCR analysis on cotyledon RNA confirmed that *COL6* and *COL16* are indeed down-regulated in the *ppi2-2* mutants (Fig. 2). In contrast, up-regulation rather than down-regulation of most other genes in *ppi2-2* was observed (Fig. 2). The discrepancy between the SuperSAGE and real-time PCR results may be attributed to the difference in the developmental stages studied in these experiments. It has been suggested that plastid signalling pathways observed in young seedlings are not the same as those in older plants (Pogson *et al.*, 2008). For example, the effect of Lin on nuclear gene expression is observed only within a few days after germination (Sullivan and Gray, 1999). While 5-day-old seedlings were used in this study, rosette leaves harvested from 2-week-old plants were used in the SuperSAGE analysis (Kakizaki *et al.*, 2009). In wild-type plants treated with NF or Lin, the responses of these transcription factor genes were similar to those observed in the *ppi2-2* mutants, although there were a few exceptions (Fig. 2). Therefore, the changes in expression of these transcription factors, at least when examined at the cotyledon stage, are similar for the three different treatments.

To investigate further the effect of different treatments on the expression of nuclear genes, the 1000 most strongly up-regulated genes in *ppi2-1* identified in a previous SuperSAGE analysis (Kakizaki *et al.*, 2009) were chosen and their expression profiles were investigated in NF- or Lin-treated plants by using the available microarray data set. As shown in Supplementary Fig. S1 at *JXB* online, the majority of these genes were not affected by the inhibitor treatments, implying that the three plastid signals act differently on the expression of nuclear genes. However, these large-scale data

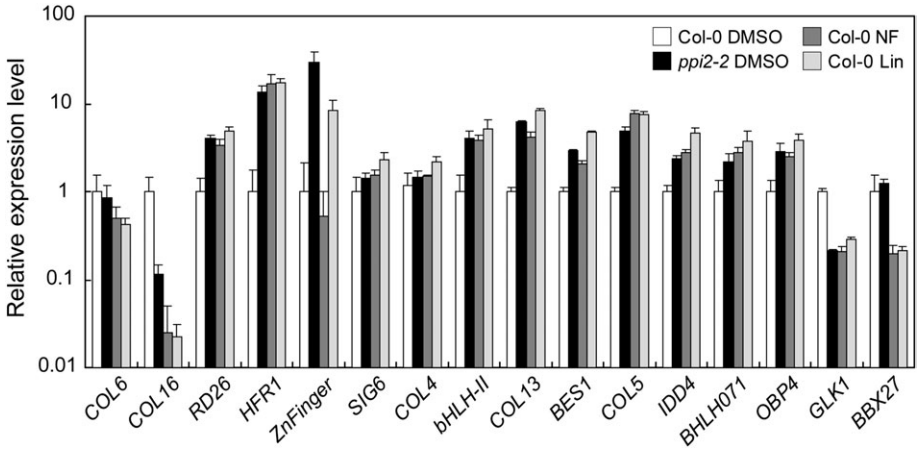

Fig. 2. Expression profiles of transcription factor-encoding genes in *ppi2-2* mutant and wild-type plants treated with either 1 μM norflurazon (NF) or 500 μM lincomycin (Lin). Transcript levels were analysed by real-time PCR and normalized to the level of *ACTIN2*. Each bar is plotted on a log scale and represents the mean of at least three independent samples. Error bars represent the standard deviations of the mean. Transcription factors that are down-regulated in true leaves of *ppi2-1* mutant plants (Kakizaki *et al.*, 2009) were analysed. AGI codes are listed in Supplementary Table S1 at *JXB* online.

were not obtained from plants grown under the same conditions or at the same developmental stage. Therefore, the expression of several of these transcription factors was analysed quantitatively by real-time PCR using mutants and inhibitor-treated plants grown under the same conditions. According to the analysis shown in Supplementary Fig. S1, these transcription factors are significantly up-regulated in *ppi2-2* mutants but down-regulated by NF treatment. Real-time PCR confirmed that most of these genes are indeed up-regulated in the *ppi2-2* mutants (Fig. 3). However, in contrast to the implications of the microarray data, most of these transcription factor genes were also up-regulated by NF or Lin treatment; that is, they responded similarly to all three treatments. Although the responses (e.g. of the genes *PIF3*, *SEP3*, *jmjC*, and *ERF6*) to the different treatments were not equally strong, they were at least qualitatively similar. Therefore, the results suggest that the changes in nuclear gene expression caused by the *ppi2-2* mutation are somewhat similar to those observed after treatment with the inhibitors.

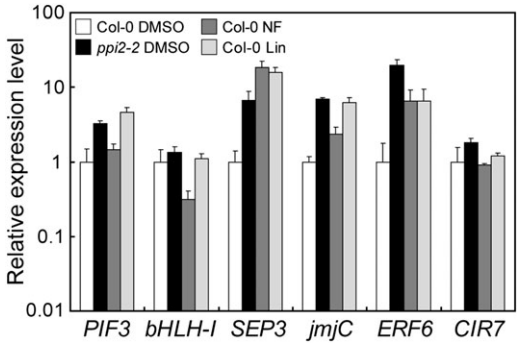

Fig. 3. Expression profiles of several other transcription factors in *ppi2-2* and wild-type plants treated with either 1 μM norflurazon (NF) or 500 μM lincomycin (Lin). Transcription factors were selected based on comparative analysis of SuperSAGE results and available microarray data sets (Supplementary Fig. S1 at *JXB* online). Transcript levels were analysed by real-time PCR and normalized to the level of *ACTIN2*. Each bar is plotted on a log scale and represents the mean of at least three independent samples. Error bars represent standard deviations of the mean. AGI codes are listed in Supplementary Table S1 at *JXB* online.

Inhibitor treatments and the ppi2-2 *mutation result in defective RNA editing of* accD *and* rps14

The gene expression analysis suggested that the two treatments and the *ppi2-2* mutation show similarities in their plastid signalling and cause similar transcriptional responses in the nucleus. Therefore, investigations were carried out to determine whether similar plastid processes are affected by the two treatments and the *ppi2-2* mutation. First the plastid processes that are affected by the *ppi2-2* mutation were explored, focusing on RNA metabolism in plastids. A unique process that occurs in plastids is RNA editing. In these organelles, RNA editing converts specific cytidine residues to uridines (Stern *et al.*, 2010). This process frequently generates amino acid substitutions in protein-coding sequences and sometimes creates start or stop codons. The fact that some mutants with defects in chloroplast RNA editing exhibit a pale or albino phenotype (Yu *et al.*, 2009; Tseng *et al.*, 2010) led to the investigation of whether chloroplast RNA editing is affected by the *ppi2-2* mutation.

The *accD* and *rps14* genes were first investigated. The *accD* gene encodes the β-subunit of acetyl-CoA carboxylase and is involved in lipid biosynthesis (Sasaki *et al.*, 1993). A serine-to-leucine amino acid substitution caused by RNA editing was predicted to be required for normal acetyl-CoA carboxylase activity in *Arabidopsis* (Sasaki *et al.*, 2001). The *rps14* gene encodes the chloroplast ribosomal protein S14 (Shinozaki *et al.*, 1986). Based on their functions, these two genes are very likely to be essential for plastid function.

Therefore, it was hypothesized that defective editing of these essential genes might be associated with the severe growth defects of NF- and Lin-treated and *ppi2-2* mutant plants. As shown in Fig. 4A, the *ppi2-2* mutants showed defects in RNA editing of *accD* and *rps14* transcripts. Next examinations were conducted to determine if RNA editing is impaired in the *gun1* mutant. Previous genetic studies have indicated that the *gun1-101 ppi2-2* double mutant has an embryonic lethal phenotype (Kakizaki *et al.*, 2009). Therefore, the possibility that the *gun1* mutation further impaired RNA editing of *accD* and *rps14* in plastids, which would explain the lethality of the double mutation, was explored. RNA editing of *accD* and *rps14* in the *gun1-101* single mutants was normal (Supplementary Fig. S2 at *JXB* online). In contrast, RNA editing of *accD* and *rps14* was defective in the *gun1-101 ppi2-2* double mutants, and the editing efficiencies of these genes were similar to those observed in the *ppi2-2* single mutants (Fig. 4A). These results indicate that the *ppi2-2* mutation affects RNA editing of *accD* and *rps14* and that GUN1 is not involved in this process.

Given the fact that the *ppi2-2* mutation affected RNA editing of *accD* and *rps14*, the effects of NF and Lin treatments on chloroplast RNA editing were also investigated. Lin and NF have been widely used to investigate plastid signalling pathways. Lin is a specific inhibitor of protein synthesis in plastids, whereas NF inhibits phytoene desaturase (Chamovitz *et al.*, 1991; Mulo *et al.*, 2003).

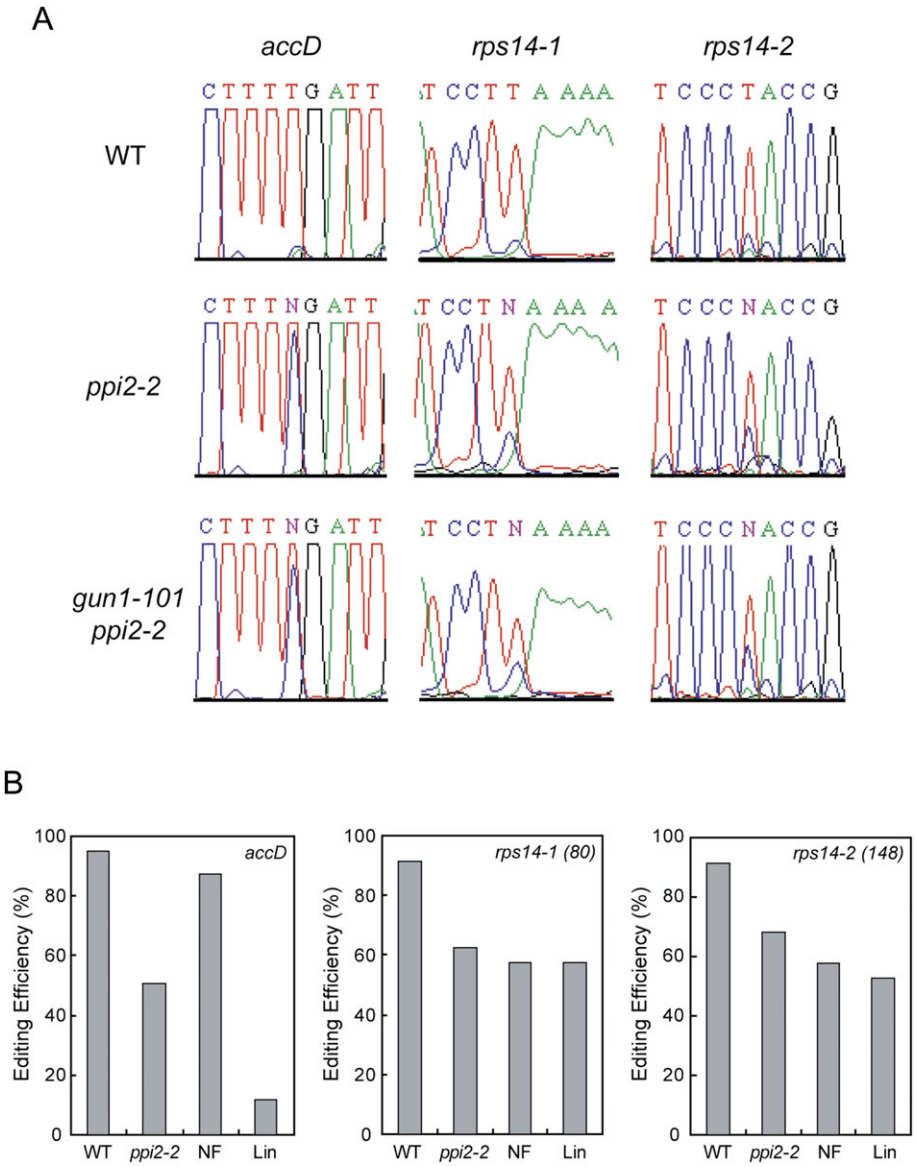

Fig. 4. Analysis of chloroplast RNA editing. (A) Comparison of the RNA editing efficiencies of *accD* and *rps14* in *ppi2-2* and *gun1-101 ppi2-2* mutant plants. RT-PCR products were sequenced directly to determine their editing efficiencies. (B) Quantitative analysis of the RNA editing efficiencies of *rps14* and *accD* transcripts. RT-PCR products were subcloned into the pZErO-2 vector and their sequences analysed. At least 99 independent clones were sequenced for each treatment. WT, wild-type treated with DMSO; *ppi2-2*, *ppi2-2* mutant treated with DMSO; NF, wild-type treated with norflurazon; Lin, wild-type treated with lincomycin.

Although it has been shown that antibiotics that inhibit plastid translation also selectively inhibit RNA editing in plastids (Karcher and Bock, 1998), it remains to be determined if this phenomenon is associated with plastid signalling. To examine the RNA editing efficiencies of *rps14* and *accD* in a quantitative manner, cDNAs were synthesized from RNA of NF- and Lin-treated wild-type plants and untreated *ppi2-2* mutant plants and cloned into a vector. At least 99 independent clones were sequenced and the percentage of correct RNA editing was estimated for each treatment. As shown in Fig. 4B, RNA editing of *rps14* transcripts was significantly impaired in NF- and Lin-treated plants. The editing efficiencies of *rps14* in these plants were 50–60%, comparable with that observed in the *ppi2-2* mutants. Interestingly, the editing efficiency of *accD* in Lin-treated plants was very low, whereas nearly 100% of the *accD* transcripts were edited correctly in the NF-treated plants (Fig. 4B). These results indicate that three different treatments that generate plastid signals all resulted in defects in RNA editing of *rps14* transcripts.

Global impact of inhibitor treatments and ppi2-2 *mutation on RNA editing in plastids*

Next the analysis of RNA editing was expanded into other plastid-encoded transcripts. To date, 34 RNA editing sites have been found in plastid-encoded genes (Chateigner-Boutin and Small, 2007). Advantage was taken of a previously published method for comprehensive analysis of RNA editing in *Arabidopsis* plastids (Okuda *et al.*, 2007). cDNAs from each gene were amplified by RT-PCR from the untreated and inhibitor-treated wild-type plants and the *ppi2-2* mutant plants, and the PCR products were directly sequenced to determine editing efficiencies. As shown in Table 1, an additional 25 RNA editing sites were successfully analysed. These results demonstrated that both of the inhibitor treatments and the *ppi2-2* mutation also affect RNA editing of other transcripts (Table 1). Of particular interest, RNA editing of transcripts encoding subunits of NAD(P)H dehydrogenase, such as *ndhB*, *ndhD*, *ndhF*, and *ndhG*, was significantly impaired under all of the tested conditions. These results indicate that NF and Lin treatments and the *ppi2-2* mutation, which are known to induce plastid signals, have global impacts on RNA editing in plastids.

Effects of NF, Lin, and the ppi2-2 *mutation on gene expression of plastid-localized PPR proteins*

The RNA editing defect in NF- and Lin-treated plants and in the *ppi2-2* mutant plants prompted testing of the nuclear expression of *PPR* genes in these plants. Plastid-localized PPR proteins are known to be involved in RNA metabolism, including RNA editing (Schmitz-Linneweber and Small, 2008). It has been shown that the PPR proteins VAC1/ECB2 and RARE1 are involved in the editing of *accD* (Robbins *et al.*, 2009; Yu *et al.*, 2009; Tseng *et al.*, 2010). Likewise, other mutations of *PPR* genes have been shown to affect

Table 1. Summary of the effects of NF and Lin treatments and the *ppi2-2* mutation on RNA editing within plastids

++, more than 70% of transcripts were edited; +, 30–70% of transcripts were edited; –, less than 30% of transcripts were edited.

	Col-0	*ppi2-2*	NF	Lin
atpF	++	++	++	+
clpP	++	+	++	+
matK	++	++	++	++
ndhA	–	–	–	–
ndhB-1	++	+	–	–
ndhB-2	++	–	–	–
ndhB-3	++	–	–	–
ndhB-7	++	+	+	–
ndhB-8	++	–	–	–
ndhB-9	++	–	–	–
ndhB-11	++	–	–	–
ndhB-12	++	–	–	–
ndhB-10	++	–	+	–
ndhD-2	++	++	+	+
ndhD-4	++	+	++	+
ndhD-3	++	+	–	–
ndhD-5	++	+	++	+
ndhF	++	+	+	–
ndhG	++	–	+	–
psbE	++	++	++	++
psbF	++	++	++	+
petL	++	+	++	++
rpoB-1	++	++	++	+
rpoB-3	++	++	++	++
rpoB-7	++	++	++	++

RNA editing of specific transcripts (Hammani *et al.*, 2009). Therefore, the expression of 11 plastid-localized PPR protein genes was examined (Fig. 5). These *PPR* genes have been shown to affect RNA editing of at least one of the impaired transcripts listed in Table 1 (Hammani *et al.*, 2009). As shown in Fig. 5, the expression levels of *VAC1*/*ECB2* and *RARE1* were up-regulated by NF and Lin treatments and the *ppi2-2* mutation. Other PPR proteins were also examined, such as *CRR22*, *CRR28*, *CRR21*, and *CRR4*. These proteins are known to affect RNA editing of *ndhB* and *ndhD* transcripts in chloroplasts (Okuda *et al.*, 2007, 2009). Therefore, it was possible that simultaneous down-regulation of *CRR* genes would impair RNA editing of *ndhB* and *ndhD* transcripts. However, except for a slight down-regulation of *CRR4* in NF- and Lin-treated plants, the expression levels of *CRR* genes were either unaffected or increased (Fig. 5). Other *PPR* genes examined were also not down-regulated in NF- and Lin-treated plants and *ppi2-2* mutant plants (Fig. 5). These data suggest that plastid signals do not significantly affect the expression levels of the *PPR* genes examined. Although the possibility that *ppi2-2* mutant and inhibitor-treated plants failed to accumulate these PPR proteins in plastids cannot be excluded, protein accumulation in the *ppi2-2* mutant (Kakizaki *et al.*, 2009) or NF-treated plants (Supplementary Fig. S3 at *JXB* online; compare with Fig. 1) was always consistent with the expression level of the corresponding mRNA, suggesting that the PPR protein

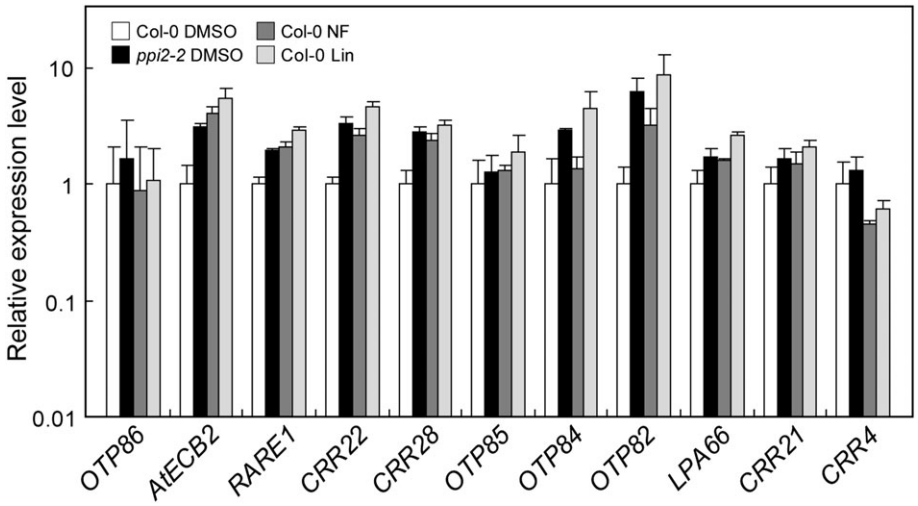

Fig. 5. Expression profiles of genes encoding pentatricopeptide repeat proteins in *ppi2-2* mutant and wild-type plants treated with either 1 μM norflurazon (NF) or 500 μM lincomycin (Lin). Transcript levels were analysed by real-time PCR and normalized to the levels of *ACTIN2*. Each bar is plotted on a log scale and represents the mean of at least three independent samples. Error bars represent standard deviations of the mean. AGI codes are listed in Supplementary Table S1 at *JXB* online.

accumulation is likely to be normal in plastids for the proteins examined. Therefore, the defects in RNA editing resulting from the NF and Lin treatments and the *ppi2-2* mutation do not seem to be caused by insufficient nuclear expression of the *PPR* genes. It is more likely that the inhibition of plastid biogenesis by the three different treatments affects RNA editing in plastids.

Discussion

Plastid signals are proposed to play crucial roles in plant development. However, whether plastid signals truly exist as such has been a matter of debate for years, and none of the actual signalling factors has been identified in land plants (Pfannschmidt, 2010). To elucidate further the nature of plastid signalling, the gene expression profile of *ppi2-2* mutant plants was compared with that of wild-type plants treated with NF or Lin. The data suggest that plastid signals resulting from these treatments have similar effects at the gene expression level (Figs 1–3). Unexpectedly, it was found that RNA editing of *accD* and *rps14* is partially impaired in the *ppi2-2* mutants (Fig. 4). This effect on plastid RNA editing is likely to be associated with plastid signalling, as NF and Lin treatments and the *ppi2-2* mutation all globally affected RNA editing in plastids (Fig. 4 and Table 1). Antibiotics inhibiting plastid translation have previously been shown to selectively inhibit RNA editing in plastids (Karcher and Bock, 1998), but no possible link between plastid signalling and RNA editing has been proposed to date. Since the expression levels of *PPR* genes involved in the editing of impaired transcripts were not decreased in the affected plants, the RNA editing phenotype does not seem to be due to secondary effects related to lower expression levels of those genes. The data suggest an unprecedented link between RNA editing and plastid signalling.

Whether a defect in RNA editing itself can act as a plastid signal remains to be established. In NF-treated wild-type plants, the editing of the *accD* transcript was normal (Fig. 4B), suggesting that the impairment in RNA editing of *accD* is itself less likely to act as a direct plastid signal. Consistent with this idea, a lack of editing of *accD* did not affect plant growth in the *rare1* mutant (Robbins *et al.*, 2009). It is also conceivable that incomplete Rps14 protein resulting from incomplete editing of the transcript affects the synthesis of plastid-encoded proteins, thereby influencing metabolic processes involved in the production of plastid signals. However, the lack of plastid ribosomes in barley mutant *albostrians* and maize mutant *iojap* did not affect RNA editing in plastids (Zeltz *et al.*, 1993; Halter *et al.*, 2004). Since the *albostrians* mutation is also known to generate plastid signals (Bradbeer *et al.*, 1979), an additional explanation is necessary. One possible scenario is that a dysfunctional NAD(P)H dehydrogenase (NDH) complex in plastids is responsible for the generation of plastid signals by each treatment. Among the 16 editing sites within *NDH* transcripts that were examined (Table 1), at least 14 were affected by inhibitor treatments and the *ppi2-2* mutation. In chloroplasts, the NDH complex participates in the cyclic electron transport around photosystem I and also plays a role in alleviating over-reduction in the stroma under stress conditions (Peng *et al.*, 2011). Therefore, it is reasonable to speculate that alteration of NDH activity by impaired RNA editing could affect the redox state of plastids in treated plants, thereby triggering plastid signal transduction. Alternatively, the global impact on RNA editing, rather than impaired RNA editing of specific transcripts, may be important for plastid signalling. This hypothesis would explain the quantitatively different effects of each treatment on nuclear gene expression (Figs 1–3, 5). Consistent with this idea, Lin treatment indeed has the greatest impact on both RNA editing and photosynthesis-related gene expression (Fig. 1 and, Table 1).

It has been postulated that distinct signalling molecules are generated by each treatment that induces plastid signals (Woodson and Chory, 2008). However, it is also conceivable that an as yet uncharacterized common signalling molecule exists for multiple treatments. It is demonstrated that three different conditions, a protein import defect, Lin treatment, and NF treatment, all of which are known to induce plastid signals, also affect RNA editing in plastids. This finding implies that different treatments potentially affect similar, if not identical, plastid processes, thereby generating a common plastid signalling molecule. Indeed, this idea is consistent with the fact that plastid-localized GUN1 plays a central role in signalling in response to several different plastid signal-inducing treatments (Koussevitzky *et al.*, 2007; Kakizaki *et al.*, 2009). Based on an analysis of the *vac1* mutant, which is defective in RNA editing of *accD*, a plastid signal generated by this mutant has been postulated to be independent of GUN1 (Tseng *et al.*, 2010). It was found that NF and Lin treatments, as well as the *ppi2-2* mutation, affected RNA editing in plastids (Table 1) but that the signals induced by these treatments were mediated by GUN1 (Koussevitzky *et al.*, 2007). Therefore, a model is favoured in which GUN1 mediates a plastid signal that is associated with impaired RNA editing. Intriguingly, the *gun1* mutation neither affected RNA editing of the chloroplast genes that were examined nor exacerbated the reduced editing efficiencies in the *ppi2-2* mutant background (Fig. 4B). If GUN1 is part of this elusive signalling pathway, defects in RNA editing or in other plastid processes that are affected by the different treatments in a similar way might utilize this protein as a downstream signalling component. It has been shown that inhibitor treatments induce the production of reactive oxygen species (Moulin *et al.*, 2008; Saini *et al.*, 2011). A mutant defective in thylakoidal protein translocation also suffered from oxidative stress (Liu *et al.*, 2010). Therefore, the possibility cannot be excluded that other plastid processes, such as the production of reactive oxygen species, may act as a source of common plastid signals. Further exploration of such plastid processes would help reveal the nature of plastid signals.

It is emphasized that it is still unclear whether the defect in RNA editing in plastids acts as part of the plastid signalling pathway. Nonetheless, the results pointed out a potential problem with the current model of the plastid signalling pathway. Inhibitors have been used to analyse this pathway for many years (Pfannschmidt, 2010). These reagents are believed to affect specific pathways in plastids. For instance, NF is used as a specific inhibitor of carotenoid synthesis, while Lin inhibits the activity of a plastid-localized ribosomal protein (Chamovitz *et al.*, 1991; Mulo *et al.*, 2003). Nonetheless, it was found here that both Lin and NF partially impaired RNA editing in plastids (Fig. 4 and Table 1). While inhibitors are important tools for assessing biological phenomena, they sometimes cause side effects. For example, 2,3,5-triiodobenzoic acid has been used as an inhibitor of polar auxin transport (Morris, 2000). It does indeed affect polar auxin transport by inhibiting trafficking of the auxin efflux carrier PIN1, but it also affects the trafficking of a plasma membrane ATPase (Geldner *et al.*, 2001). Given these observations, it would not be surprising if secondary effects of these inhibitors generate 'true' plastid signals. The mechanism by which inhibitors and the *ppi2-2* mutation affect RNA editing remains to be characterized. If the RNA editing machinery in plastids contains a plastid-encoded subunit, one could speculate that the inhibitors and the *ppi2-2* mutation affect biosynthesis of this subunit. Several recent studies have also focused on interactions between inhibitor-induced plastid signals and other signalling pathways (Ruckle and Larkin, 2009; Cottage *et al.*, 2010; Voigt *et al.*, 2010). These approaches might help uncover the nature of plastid signals.

In summary, it was shown that different treatments that induce plastid signals result in defects in a common plastid process: RNA editing. Although direct evidence for a causal relationship between incomplete RNA editing and plastid signals is lacking, the results suggest an unprecedented link between the plastid signalling pathway and RNA editing.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Comparison of the gene expression profile in the *ppi2-1* mutant with those in norflurazon (NF)- and lincomycin (Lin)-treated wild-type plants.

Figure S2. Analysis of chloroplast RNA editing in the *gun1-101* mutant.

Figure S3. Effects of NF treatment on the accumulation of plastid proteins.

Table S1. Gene-specific primers used in real-time PCR.

Table S2. Primers used in RNA editing analysis.

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