The Arabidopsis gene YS1 encoding a DYW protein is required for editing of rpoB transcripts and the rapid development of chloroplasts during early growth

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Summary

Virescence, a phenotype in which leaves green more slowly than usual, is recognized to play a role in protection from photo-oxidative damage before healthy chloroplasts are developed. The elucidation of the molecular mechanisms underlying virescence will provide insights into how the development of chloroplasts is controlled. In this study, we find that knockout alleles of Yellow Seedlings 1 (YS1) in Arabidopsis lead to a virescent phenotype, which disappears by 3 weeks after germination. The ys1 mutation resulted in marked decreases in photosynthetic capacity and photosynthetic pigment complexes, and disturbed ultrastructure of thylakoid membranes in 8-day-old seedlings. However, cotyledons of ys1 seedlings pre-treated in the dark for 5 days turn green almost as fast as the wild type in light, revealing that the developmental defects in ys1 are limited to the first few days after germination. Inspection of all known plastid RNA editing and splicing events revealed that YS1 is absolutely required for editing of site 25992 in rpoB transcripts encoding the beta subunit of the plastid-encoded RNA polymerase (PEP). YS1 is a nuclear-encoded chloroplast-localized pentatricopeptide repeat protein differing from previously described editing factors in that it has a C-terminal DYW motif. A defect in PEP activity is consistent with the changes in plastid transcript patterns observed in ys1 seedlings. We conclude that the activity of PEP containing RpoB translated from unedited transcripts is insufficient to support rapid chloroplast differentiation.

Keywords: chloroplast development, pentatricopeptide repeat protein, plastid gene expression, RNA polymerase, Arabidopsis.

Introduction

The virescent phenotype is defined as presenting young leaves with reduced chlorophyll levels but can accumulate almost normal amounts of chlorophyll as they mature (Archer and Bonnett, 1987). Due to the unique features of their chloroplast biogenesis and potential value in agriculture, virescent mutants have been studied for more than half a century in various plant species, such as maize (Zea mays; da Costa e Silva et al., 2004), rice (Oryza sativa; Kusumi et al., 1997; Sugimoto et al., 2007), cotton (Gossypium; Rhine, 1955), tobacco (Nicotiana tabacum; Archer and Bonnett, 1987), peanut (Arachis hypogaea; Benedict and Ketting, 1972) and Arabidopsis (Loschelder et al., 2006; Koussevitzky et al., 2007). To date, however, only a few of the genes responsible for virescent phenotypes have been isolated and their molecular action remains largely unknown.

Plastids of higher plants arose from cyanobacteria through endosymbiosis and have their own genome (Dyall et al., 2004) containing around 40 photosynthesis-related genes and 60 housekeeping genes that are essential for protein synthesis (Sato et al., 1999). It is well documented that coordination of nuclear and plastid gene expression is crucial for chloroplast biogenesis (Mullet, 1988). Transcript-
tion of the plastome relies on two types of plastid RNA polymerases: nucleus-encoded RNA polymerase (NEP) that resembles those of the T7 bacteriophage (Hedtke et al., 1997), and plastid-encoded RNA polymerase (PEP), which is a multi-subunit eubacterial-type enzyme (Hess and Börner, 1999). Nucleus-encoded polymerase predominantly transcribes housekeeping genes (e.g. rpoB, accD) (Hajdukiewicz et al., 1997), whereas PEP is responsible for transcription of photosynthesis-related genes (e.g. rbcL, psbA) (Liere and Maliga, 1999). Promoter specificity and transcription initiation by PEP requires nucleus-encoded proteins, such as σ-like factors whose expression is spatially and temporally regulated by environmental cues (Allison, 2000; Kasai et al., 2004; Ishizaki et al., 2005). At the beginning of light-induced chloroplast development, NEP is imported into plastids and initiates transcription of housekeeping genes including PEP-encoding genes, followed by an increase in PEP-dependent transcription (Hajdukiewicz et al., 1997). Control of plastocline expression has been demonstrated at various levels, such as transcription, RNA processing and stability, and translation (Barkan and Goldschmidt-Clermont, 2000).

Several lines of evidence have demonstrated that the virescent phenotype is closely associated with reduced activity of PEP or NEP at an early stage of chloroplast development. In Arabidopsis, mutations in SIG6 cause a weakly virescent phenotype, with only the first pair of leaves lacking normal pigment levels (Loschelder et al., 2006). Transcripts of several PEP-dependent plastid genes are markedly and specifically reduced in the sig6 mutant (Loschelder et al., 2006). Partial silencing of one of the NEP genes, RPOTP, leads to a typical virescent phenotype of Arabidopsis seedlings, which can become as green as the wild type after 2 weeks of growth (Swiatecka-Hagenbruch et al., 2008). A knockout of RPOTP, however, exhibits severe deficiency in chloroplast biogenesis as well as in cell proliferation (Hricová et al., 2006). Very severe delays to normal greening are also seen in clb19 mutants, in which RNA editing of rpoA transcripts is lacking leading to a loss in activity of PEP (Chateigner-Boutin et al., 2008). CLB19 is one of many nuclear pentatricopeptide repeat (PPR) genes required for proper organellar gene expression. At least 450 PPR genes exist in the Arabidopsis genome (Aubourg et al., 2000; Lurin et al., 2004; Rivals et al., 2006). They are characterized by tandem 35-amino-acid motifs, which share some features with the better-known tetratricopeptide repeat (TPR) motif (Small and Peeters, 2000). Pentatricopeptide repeat proteins are thought to bind DNA or RNA molecules and/or to mediate protein–protein interactions (Delannoy et al., 2007). In general, PPR proteins have been implicated in RNA transcription (Ikeda and Gray, 1999; Pflaz et al., 2006), RNA editing (Kotera et al., 2005; Okuda et al., 2007; Chateigner-Boutin et al., 2008), RNA processing (Meierhoff et al., 2003; Hattori et al., 2007), RNA splicing (Schmitz-Linneweber et al., 2006; Falcon de Longevialle et al., 2007, 2008), RNA stability (Beick et al., 2008) and translational activation (Schmitz-Linneweber et al., 2005; Tavares-Carreón et al., 2008). However, most PPR genes remain to be investigated with regard to their physiological functions, biochemical properties and mechanism of action. Here, we show that a chloroplast-localized PPR protein, YS1, is required for the editing of rpoB transcripts in Arabidopsis. YS1 is particularly interesting because it is a member of the DYW subgroup of the PPR family, suggested on phylogenetic grounds to be likely editing factors (Salone et al., 2007; O’Toole et al., 2008) but for which experimental proof was lacking. Lack of editing of rpoB transcripts leads to a significant decrease in PEP activity and, in turn, a delay in the rapid development of chloroplasts during early growth.

Results

Identification and characterization of the ys1 mutant

In order to gain more insight into the role of PPR proteins in early chloroplast development, we screened for virescent mutants with slow greening of cotyledons and/or leaves from a batch of Arabidopsis PPR mutants ordered from the SALK T-DNA collection (Alonso et al., 2003). In this study, we investigated a virescent mutant (Salk_123515), designated yellow seedling 1 (ys1-1), because the chlorophyll content of the mutant was ultimately able to completely restore to the wild-type level. The virescent phenotype of ys1-1 was imaged at day 4 (Figure 1a), day 8 (Figure 1b), day 12 (Figure 1c) and day 25 (Figure 1d) after germination. This observation was consistent with the time-course changes in chlorophyll and carotenoids in mutant and wild-type plants (Figure 1e). In contrast, the leaf area of the mutant reached only about 55% of the wild type even at day 30 after germination (Figure 1f). Leaves emerging from more than 4-week-old mutant plants could reach the same chlorophyll level as those of the wild type within 2 days, and mature plants were almost indistinguishable between the mutant and the wild type in terms of rosette diameter, bolting time and seed fertility (data not shown).

YS1 encodes a chloroplast-localized PPR protein

Our genetic analysis showed that the virescent phenotype of ys1-1 was controlled by a recessive nuclear gene and co-segregated with the kanamycin resistance marker (data not shown). Plasmid rescue and sequencing of the PCR products showed that the T-DNA was inserted in the first exon, 378 bp downstream of the start codon, of the Arabidopsis gene At3g22690 (henceforth named YS1). Analyses of the deduced YS1 amino acid sequence revealed a predicted chloroplast transit peptide at its N terminus, followed by 16 PPR motifs and E and DYW motifs (Figure 1g). The predicted gene model for YS1 in the TAIR
YS1 is required for editing of rpoB transcripts

Figure 1. Identification and characterization of the ys1 mutant.
(a) Four-day-old plants; (b) 7-day-old plants; (c) 12-day-old plants; (d) 25-day-old plants. (e) Time course of pigment accumulation. (f) Change in leaf area.
(g) Localization of the T-DNA insertions in the three mutant alleles and arrangement of the various motifs within the YS1 sequence. Arrows represent the position and orientation of the gene-specific primers used in (h) and (i). (h) Identification of the T-DNA insertions in the ys1 mutants by PCR with genomic DNA as template. (i) Messenger RNA of YS1 detected by RT-PCR. The actin transcript from the same cDNA preparations was used as an internal control. (j) Detection of YS1 transcripts in various tissues. Total RNA was extracted from the indicated tissues of 8- or 30-day-old light-grown, or 5-day-old etiolated plants.
alleles (Figure 1i). In the wild type, et al. (2008), doi: 10.1111/j.1365-313X.2008.03766.x

Loss of YS1 leads to low photosynthetic capacity

To understand the physiological role of YS1 in chloroplast development, we characterized the photosynthetic capacity of the mutant. Chlorophyll fluorescence induction experiments showed that the ratio of variable fluorescence to maximum fluorescence ($F_v/F_m$), which reflects maximum photochemical efficiency of photosystem II (PSII), was significantly lower in ys1 ($0.20 \pm 0.02$) than in the wild type ($0.78 \pm 0.01$) (Figure 2a and Table 1). This was supported by the quantum yield of PSII ($\Phi_{PSII}$), with 0.12 $\pm 0.01$ in ys1 versus 0.56 $\pm 0.02$ in the wild type (Table 1). Loss of YS1 also led to a significant decrease in the electron transport rate (ETR), the relative flow of electrons through PSII (Table 1). The reduced ETR was coincident with the enhanced $1–q_P$ value, a parameter of the oxidation state of the plastoquinone $Q_A$ of PSII, indicating that $Q_A$ was in a reduced state and electron flow was impaired downstream of $Q_A$ in ys1. To find out whether ETR was also blocked at PSI, we determined P700 redox kinetics under far-red light. Our data showed that the mutant had significantly lower PSI activity relative to the wild type (Figure 2b). Additionally, $\Delta A/\Delta A_{max}$ was significantly increased (Table 1), indicating that electron transport was also restricted in the intersystem chain.

We further examined the status of PSI and PSII using 77 K chlorophyll fluorescence emission spectra from seedlings. Compared with the highly reduced level of fluorescence emission from PSI (with a maximum at 735 nm), the magnitude of the fluorescence emission from PSII was not obviously altered by YS1 mutation (Figure 2c). The signal of the high-wavelength band indicated a nearly normal accumulation of the outer antenna of PSII, suggesting that the accumulation of the PSI core complex was more decreased than PSII in ys1. However, a blue shift of the characteristic PSII fluorescence peak at 687 nm and 695 nm was observed in the mutant, indicating that excitons were unable to be transferred efficiently to the PSII reaction centre. A similar peak shift of the PSI fluorescence was also detected in ys1 (Figure 2c). These shifts were probably caused by partial disruption of energy transfer from the antennae to photosystems. Additionally, the ratio of $F_{PSI}$ to $F_{PSII}$ was significantly lower in ys1 relative to the wild type (Table 1). Taken together, these results clearly indicate that photosynthetic capacity is severely lowered in the ys1 mutants.

Thylakoid membranes are disturbed in ys1

To unveil the ultrastructural basis of delayed greening of ys1 seedlings, we examined dynamic changes in thylakoid membranes using transmission electron microscopy. In leaves of wild-type seedlings grown in the light, chloroplasts were crescent-shaped and contained a well-formed thylakoid structure including stroma thylakoids and grana thylakoids (Figure 3a). In contrast, at the same stage, the chloroplasts of mutant seedlings were small and thylakoid membrane structure was distorted in various ways. As shown in Figure 3(b), some plastids formed rudimentary thylakoids consisting only of grana lamellae and failed to accumulate stroma lamellae; some were filled with numerous vesicles...
Chloroplasts with abnormal thylakoid membranes were still dominant in 7-day-old leaves (Figure 3d). At day 11, chloroplasts with well-organized grana thylakoids appeared (Figure 3e), and at day 16 most chloroplasts were oval with a well-developed thylakoid structure (Figure 3f). The ultrastructure of chloroplasts was indistinguishable between \textit{ys1} and the wild type after 3 weeks’ growth (Figure 3g). Etioplasts in dark-grown cotyledons were indistinguishable between \textit{ys1} and the wild type (Figure 3h,i). These results confirm that YS1 plays an important role in the early stages of chloroplast biogenesis during leaf greening.

Accumulation of \(\delta\)-aminolevulinic acid is reduced in the light-grown \textit{ys1} mutants

Since no difference in etioplast ultrastructure was observed between \textit{ys1} and the wild type (Figure 3h,i), we reasoned that cotyledons of etiolated \textit{ys1} seedlings might be able to...
turn green like those of the wild type. To test this, 5-day-old seedlings grown in darkness were transferred to the light. The cotyledons of \( \textit{ys1} \) seedlings were able to green almost as much as the wild type 2 days after exposure to light (Figure 4a,b). However, new leaves emerging from the seedlings were still yellow (Figure 4c). These results are consistent with the time-course of changes in chlorophyll content (Figure 4d). Mutant seedlings had accumulated slightly lower levels of chlorophyll than the wild type at 12 h but contained only 59.2% of the chlorophyll of the wild type at 48 h after transfer to light (Figure 4d). We hypothesized that delayed greening of \( \textit{ys1} \) seedlings in light might result from a low level of \( d \)-aminolevulinic acid (ALA), a precursor for chlorophyll synthesis. As shown in Figure 4(e), the level
of ALA was indistinguishable between ys1 and the wild type in 5-day-old etiolated plants, whereas accumulation of ALA was significantly reduced in the mutant compared with the wild type in light-grown 5-day-old plants. These results suggest that accumulation of chlorophyll may be limited by ALA content in light-grown ys1 plants.

Assembly of photosynthetic complexes is impaired in ys1

We examined the level of photosynthetic pigment-containing complexes by blue native (BN)-PAGE and sucrose gradient centrifugation. Our data showed that PSII/light-harvesting complex (LHC) II supercomplexes, the PSII/LHCI complex and the Cyt b6/f complex were all strongly reduced in 8-day-old mutant seedlings compared with the wild type (Figure 5a,c). Additionally, ys1 accumulated less LHCII trimer but a higher level of LHCII monomer relative to the wild type (Figure 5a,c), suggesting that assembly of LHCII trimer is blocked. In the 30-day-old plants, however, no significant differences in the pigment-containing complexes were found between the mutant and the wild type (Figure 5b,d).

To understand how mutation of YS1 led to reduced photosynthetic complexes, we assayed the accumulation of representative subunits of thylakoid membrane complexes by immunoblots. Except for AtpB, the levels of plastid-encoded proteins such as D1, D2, Psaa, Psac, Cyt b6 and Cyt f were significantly reduced in ys1 compared with the wild type (Figure 6). Compared with the PSII reaction centre core subunits (D1 and D2), which were reduced to 50% of the wild type, the relative level of the PSI core protein (PsaiA) was more drastically reduced in ys1. This was consistent with the results of chlorophyll fluorescence analyses. Moreover, the reduction of Cyt b6/f subunits was also in agreement with the results from the analysis of ETR. In contrast, the nuclear-encoded subunits such as PsbO, PsaD, Lhca1 and Lhcb1 were unaltered in the mutant (Figure 6). These results suggest that the loss-of-function of YS1 specifically results in a decrease in plastid-encoded proteins.

The reduction of plastid-encoded proteins in ys1 is due to reduced levels of transcripts

Protein accumulation could be controlled at a transcriptional or post-transcriptional level. To distinguish between these two possibilities, the expression of a wide variety of plastid genes was analyzed by northern blot and quantitative RT-PCR. Results showed that mutation of YS1 caused a marked decrease in transcript accumulation of PEP-dependent photosynthesis-related (class I) genes (Figure 7a), but enhanced accumulation of transcripts of NEP-dependent housekeeping (class III) genes (Figure 7b). The 4.8-kb transcript detected with the atpB probe has been identified to be the product transiently transcribed by NEP in sig6, where NEP activity was upregulated (Schweer et al. 2006). This might explain why AtpB accumulated at the same level as that of the wild type in ys1 (Figure 6).

Consistent with protein blot analysis, nuclear gene expression was not altered in ys1 (Figure 7c). Interestingly, expression of a group of PEP-dependent trn genes including trnE significantly decreased in ys1, both in light-grown (Figure 7d) and etiolated plants (Figure 7e). The expression in etiolated plants of other PEP- or NEP-dependent genes such as psbA, psaA, petA, atpB, rbcL and accD was not radically different between ys1 and the wild type (Figure 7e).
To test whether YS1 also translationally regulates plastid gene expression, polysome-bound mRNAs were fractionated by sedimentation. The extent to which transcripts are associated with polysomes reflects the efficiency of translation initiation and elongation (Barkan, 1993). The distribution of both ribosomal RNAs and examined mRNAs was unaltered between the wild type and the mutant (Figure S4). Thus, YS1 is unlikely to mediate plastid gene expression via a translational mechanism, and we conclude that the phenotype in ys1 is due to decreased PEP activity.

YS1 is required for editing of rpoB transcripts

Several PPR proteins with C-terminal E domains have been shown to be required for editing of specific sites in Arabidopsis chloroplasts (Kotera et al., 2005; Okuda et al., 2007; Chateigner-Boutin et al., 2008). Related proteins of the DYW subgroup have also been predicted to function in RNA editing on phylogenetic grounds (Salone et al., 2007). We therefore screened for defects in RNA editing in ys1 at all 34 sites known to be edited in wild-type chloroplasts (Chateigner-Boutin and Small, 2007). A single site, at site 25992 in rpoB transcripts, was not detectably edited in light- or dark-grown ys1 seedlings or in adult (and fully green) ys1 plants (Figure 8). The two other editing sites in rpoB transcripts (and all 31 sites in other transcripts) are edited normally in ys1 (Figure S5).

Discussion

YS1 is an editing specificity factor

The loss of YS1 in ys1 mutants results in the loss of a single editing event in the rpoB transcript. Although rpoB transcripts are more abundant in ys1, and increased transcript...
levels can titrate out editing factors (Chaudhuri et al., 1995), editing of this site is undetectable in ys1 mutants (Figure 8), strongly suggesting that YS1 is absolutely required to edit this site. Furthermore, neither of the other two rpoB editing sites is affected, indicating that the 25992 site is specifically targeted by YS1. Three other protein factors have been described that are similarly required for editing of specific sites: CRR4 (Kotera et al., 2005) and CRR21 (Okuda et al., 2007), required for editing of different sites in ndhD, and CLB19, required for editing of two sites in clpP and rpoA (Chateigner-Boutin et al., 2008). The three previous factors are all similar PPR proteins within the E subclass (so-named as these proteins carry a C-terminal E domain after the PPR repeats), whereas YS1 differs in having a further C-terminal

Figure 7. Messenger RNA levels of plastid and nuclear genes encoding plastid-localized proteins in ys1 and wild-type seedlings.
Ten µg or 5 µg of total RNA was isolated from 8-old-day seedlings grown in light or from 5-day-old seedlings grown in darkness, respectively.
(a) Plastid-encoded RNA polymerase (PEP)-dependent gene transcripts in 8-old-day seedlings.
(b) Nucleus-encoded RNA polymerase (NEP)-dependent gene transcripts in 8-old-day seedlings.
(c) Nuclear gene transcripts.
(d) Transcript levels of a group of plastid trn genes.
(e) Messenger RNA levels of representative genes in each gene class in 5-day-old etiolated seedlings. The bottom of each lane shows loading controls (25S rRNA) stained with ethidium bromide.
DYW domain (Figure S6). The DYW domain was hypothesized to carry RNA editing activity based on its phylogenetic distribution (it has only ever been found in plants that carry out RNA editing, and is absent from Marchantia and green algae that do not edit organellar transcripts) and based on the presence of conserved residues that match the active site of known editing deaminases from other organisms (Salone et al., 2007). However, no genetic evidence in favour of a link between DYW proteins and editing has been published apart from a broad co-localisation between two DYW-class genes and a quantitative trait locus (QTL) for editing efficiency in a cross between two Arabidopsis accessions (Bentolila et al., 2005). The results presented here demonstrate that the DYW-containing PPR protein YS1 is an editing specificity factor for rpoB site 25992, but do not show that YS1 carries the editing activity itself; other essential factors may be required.

ys1 is an RpoB mutant

Editing at position 25992 changes codon 113 of rpoB to encode phenylalanine rather than serine. A phenylalanine codon is conserved at this position in many plant lineages including the liverwort Marchantia polymorpha that does not edit its plastid transcripts (Figure S7). This phenylalanine is located within the essential B domain of the beta subunit of the Escherichia coli RNA polymerase. Mutations in this domain are lethal in E. coli (Severinov et al., 1993). Therefore a defect in editing at this position is likely to affect RpoB function. The transcript patterns in ys1 mutants are characteristic of mutants in which PEP activity is reduced (Pfalz et al., 2006; Chateigner-Boutin et al., 2008), consistent with the primary defect being in RpoB. Deletion of rpoB in tobacco (Allison et al., 1996; De Santis-Maclossek et al., 1999) gives rise to a much stronger phenotype (total lack of photosynthetic ability and green pigmentation), so the RpoB defect in ys1 is likely to be a partial, not total, loss of activity. As transformation of Arabidopsis plastids is far from routine (Dhingra and Daniell, 2006), creating mutants of plastid genes is extremely difficult. The only similar mutant described, clb19 (Chateigner-Boutin et al., 2008), has editing defects in clpP as well as rpoA, complicating the analysis of the phenotype. ys1 mutants therefore offer a currently unique opportunity to examine the role of the PEP polymerase during Arabidopsis development.

YS1 is required for correct plastid gene expression during early chloroplast development

It is well documented that chloroplast development upon shifting from dark to light is supported by a huge increase in plastid gene expression. Both NEP and PEP are already present in dry seeds and are probably active immediately after seed imbibition (Demarsy et al., 2006). The level of NEP reaches a peak earlier than that of PEP during seed germination and early chloroplast development. The activity of NEP is reduced and maintained at a basal level during the later stages of chloroplast development, whereas at this stage PEP activity increases and is kept at a high level. PEP and NEP activities are highly coordinated to optimize plastid gene expression during light-triggered chloroplast development. Disturbance of the coordinated activities of NEP and PEP usually results in delayed or completely inhibited chloroplast development (Hajdukiewicz et al., 1997; Kusumi et al., 1997; Kanamaru et al., 2001; Ishizaki et al., 2005; Rochel et al., 2006; Pfalz et al., 2006). Recently, Hanaoka et al. (2005) reported that tRNA^Glu is a direct mediator to coordinate the activities of PEP and NEP through a feedback mechanism.

In ys1, PEP transcripts are lower in the light than in the wild type (Figure 6a,d), whilst NEP transcripts are maintained at a high level (Figure 6b). The high activity of NEP observed in ys1 is probably induced by the low accumulation of tRNA^Glu, which fails to inhibit the activity of NEP. In the dark, however, NEP and PEP transcripts, except for those of a group of trn genes, accumulate to similar levels in ys1 to those in the wild type. No obvious visible, ultrastructural or molecular phenotypic defects were noted in etiolated ys1 seedlings, indicating that although rpoB transcripts are.
edited in etiolated wild-type seedlings, this editing event is unlikely to be important for etioplast development, as opposed to the requirement for YS1-mediated rpoB editing in light-grown seedlings.

**YS1 helps to coordinate biosynthesis of chlorophyll and protein in light**

Although enzymes catalysing all reactions in the chlorophyll biosynthetic pathway have been identified in higher plants, we are still facing a challenge to understand how plants incorporate environmental and developmental cues to coordinate chlorophyll synthesis with functional chloroplast development (Tanaka and Tanaka, 2007). In the chlorophyll biosynthetic pathway, formation of ALA is the first limiting step and is regulated by multiple mechanisms. The first reaction required for ALA biosynthesis is catalysed by glutamyl-tRNA synthetase, which ligates tRNA\(^{\text{Glu}}\) to glutamate, and is shared with protein synthesis in plastids. In light-grown plants, chlorophyll biosynthesis and plastid protein synthesis are both strongly activated simultaneously. We show that under these conditions, loss of YS1 leads to decreased tRNA\(^{\text{Glu}}\) accumulation, decreased ALA and decreased chlorophyll biosynthesis. It is plausible to infer that glutamyl-tRNA\(^{\text{Glu}}\) is a rate-limiting factor for chlorophyll biosynthesis in ys1 in developing chloroplasts when protein biosynthesis competes strongly for the substrate. In etiolated seedlings the situation is different. Kleffmann et al. (2007) demonstrated that the tetrapyrrole pathway is very active in the dark, whereas photosynthesis-related metabolic pathways and plastid gene expression are only significantly activated after exposure to light. The reduced level of tRNA\(^{\text{Glu}}\) in ys1 has no effect on accumulation of ALA in etiolated seedlings, presumably because plastid protein synthesis in these conditions is greatly reduced, and no longer competes. When the seedlings are transferred to the light, chlorophyll biosynthesis can proceed without further requirement for ALA and protein synthesis can proceed rapidly without competition for glutamyl-tRNA\(^{\text{Glu}}\). This temporal uncoupling between ALA synthesis and plastid protein synthesis in etiolated seedlings transferred to the light probably explains how ys1 plants can green rapidly under these conditions.

We propose a model to explain the role of YS1 during the early development of chloroplasts (Figure 9). In this model, YS1 is required to edit rpoB transcripts to produce active PEP capable of transcribing a subclass of trn genes. The expression of the plastid trnE gene and/or other trn genes is required to meet the demand of developing chloroplasts for biosynthesis of protein and chlorophyll. Transfer RNA\(^{\text{Glu}}\) also plays an important role in feedback inhibition of NEP activity. Loss of YS1 leads to a low level of tRNA\(^{\text{Glu}}\), which limits protein and chlorophyll biosynthesis in the developing chloroplasts, and lengthens the course of chloroplast development. It will be important in the future to examine whether the extent of rpoB editing by YS1 varies according to cell type, developmental stage or environmental conditions. Such variation might indicate that the rate of rpoB editing controls expression of active PEP.

**Experimental procedures**

**Plant growth, plasmids, identification of mutants and transgenic plants**

All wild-type and mutant Arabidopsis plants were in the ecotype Columbia background. Surface-sterilized seeds were stratified for 3 days, and then sown onto half-strength Murashige and Skoog (MS) medium with 1% sucrose at 22°C under long-day (16 h light/8 h dark) conditions at 120 μmol photons m\(^{-2}\) sec\(^{-1}\).

The various plasmids were constructed using the GATEWAY cloning system (Invitrogen, http://www.invitrogen.com/). To conduct the complementation experiment, a 3778-bp YS1 genomic fragment from the ATG start codon to 472 bp downstream of the 3′ untranslated (UTR) was amplified by PCR using the primers: 5′-CACCATGGCCAGTGTTGGTGAGCATTGCT-3′ and 5′-CTAAAGTGCTTGGCTGAGGACATTGGTCT-3′. For analysis of subcellular localization of YS1, the genomic fragment encoding the N-terminal 100 amino acids (Met-1 to Phe-100) of YS1 was amplified. The resulting DNA fragments were cloned into pENTR\(^{\text{TM}}\)/SD/D-TOPO vector (Invitrogen). After sequence verification, the fragments were recombined into pGWB destination binary vectors (Research Institute of Molecular Genetics, Shimane University, Japan). The constructs were transformed into A. tumefaciens GV3101 strain and introduced into Arabidopsis mutant plants by the floral dip method (Clough and Bent, 1998). Transgenic T1 plants were identified by kanamycin resistance.
The T-DNA insertion lines SALK_123515 (ys1-1), SALK_144420 (ys1-2) and SALK_073746 (ys1-3) were obtained from the Arabidopsis Biological Resource Center (ABRC; Ohio State University). Plasmid rescue was conducted as described by Ichikawa et al., 2003. Primers used for genomic PCR verification of T-DNA insertion in the mutants were 5'-CGCAGCTTAAACATCGAAATATC-3' and 5'-GGCGATGTGGTGAAATGTTCTTTCC-3'. The left border primer used was 5'-GGCTTAGGACGCCCTTGCTTCGAACT-3'. The plants homozygous for T-DNA insertion were checked for lack of YS1 transcripts using primers 5'-TCTGTTGTTAGCCGATAGATTCTC-3' and 5'-AG-TCTGACATCAAGCTGAATTCGGTC-3' by RT-PCR.

### Pignots and ALA analysis
Chlorophylls and total carotenoids were estimated spectrophotometrically according to Lichtenthaler (1987). For ALA determination, seedlings grown for 7 days on 1/2 MS medium containing 1% sucrose were incubated with 50 mM levulinic acid (LA) in 0.1 M sodium phosphate buffer (pH 7.0) in the light or the dark for 12 h. The method of ALA measurement was as previously described (Kruse et al., 1997). Measurements were repeated in three independent experiments.

### Chlorophyll fluorescence induction, P700 redox kinetics and 77 K fluorescence emission spectra
**In vivo** chlorophyll a fluorescence measurements were performed with 8-day-old Arabidopsis seedlings grown in the light at room temperature using a pulse amplitude-modulated fluorometer equipped with a PDA100 data acquisition system to record fast changes (PAM 101, Walz, Germany). Prior to measurements, the fibre optic of the emitter/detector unit (101-ED) was positioned close to the upper surface of the plants. Consecutive saturating light pulses of 800 msec were applied by halogen lamps. The connected emitter–detector subunit allowed us to measure the light-induced P700 redox kinetics by absorbance changes at 830 nm. Chlorophyll fluorescence emission spectra of ground leaves at 77 K were recorded as described (Meurer et al., 1996a,b).

### Microscopy
Wild-type and mutant leaves from different growth stages of plants were collected for transmission electron microscopy processing. The tissue was cut into small pieces and fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 4 h at 0°C. After fixation, the tissue was rinsed and post-fixed overnight at 4°C in 1% OsO4. After rinsing in phosphate buffer, the samples were dehydrated in an ethanol series, infiltrated with a graded series of epoxy resin in epoxy propoxane, and embedded in Epox 812 resin. Thin sections were stained in uranyl acetate followed by lead citrate and viewed with a transmission electron microscope (Phillips CM120, http://www.fei.com). The GFP fluorescence was observed with a confocal laser scanning microscope (FITC488, Zeiss LSM500, http://www.zeiss.com/). The filter sets used were BP505-545 (excitation 488 nm; emission 505–545 nm) and LP585 (excitation 488 nm; emission 585 nm) to detect GFP and the chlorophyll autofluorescence.

### Separation of thylakoid membrane complexes
The leaves were homogenized in an ice-cold isolation buffer containing 400 mM sucrose, 50 mM 2-aminomethyl-1,3-propanediol (TRIS)-HCl, pH 7.6, and 10 mM NaCl with a chilled mortar and pestle and filtered through two layers of nylon cloth. The filtrate was centrifuged at 5000 g for 10 min. The thylakoid pellets were washed with isolation buffer, recentrifuged and suspended in isolation buffer. The resulting thylakoid membrane pellets were either used freshly or frozen in liquid N2 and stored at −70°C before use. The chlorophyll content was determined spectrophotometrically according to Porra et al. (1989). Thylakoid membrane complexes were separated by BN-PAGE (Schagger et al., 1994; Cline and Mori, 2001) or by sucrose density gradient centrifugation (Swiatek et al., 2003).

### Immunoblot analysis
Isolated thylakoid samples were separated by 15% SDS-PAGE, transferred to Hybond-ECL Nitrocellulose membrane (Amersham Biosciences, http://www.amersham.com/), and immunoblotted with various antibodies (Agrisera, http://www.agrisera.com/). Antibodies were detected using a chemiluminescence detection system (ECL; Amersham Biosciences) according to the manufacturer’s instructions.

### Analyses by RT-PCR and northern blot
Total RNA was isolated using the RNAagents total RNA isolation system (Promega, http://www.promega.com/). Semi-quantitative RT-PCR was carried out using a reverse transcription system (Promega). The following gene-specific primers were used: the Arabidopsis Actin2 gene (At3g18780) was amplified using primers 5’-TCTTCTTTCGCTCTTTCTCTTC-3’ and 5’-TCTTACATTTCGCCCTTCGCTC-3’ as an internal positive control. Amplified DNA products were separated using an agarose gel followed by image analysis with GIS-2010 (Tanon, http://www.bio-tanon.com.cn). Northern blot analysis was carried out as described (Huang et al., 2000). The sequences of the PCR primers used for amplifying the genes are presented in Table S1. The plastome-wide screen of RNA editing was carried out as described by Chateigner-Boutin and Small (2007). The results of screening for RNA editing and splicing were confirmed by sequencing. The editing sites were amplified by PCR with primers rpoB-F2 (5’-CACCATCCTGGGATGAAAGA-3’) and rpoB-R2 (5’-TAAACCTTCCTCTATTAACCTGA-3’). The RT-PCR products were sequenced directly.

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### Supporting Information
Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Open reading frame of YS1 deduced from 3’-rapid amplification of cDNA ends (RACE) experiments.

**Figure S2.** Alignment of YS1 homologues from other species.

**Figure S3.** Subcellular localization of YS1 estimated by GFP-tagging in mesophyll cells.

Figure S4. Association of several chloroplast mRNAs with polysomes in \( \gamma S \) and wild-type seedlings.

Figure S5. Complete screen of editing sites in \( \gamma S \) mutants.

Figure S6. Alignment of the YS1 sequence with that of the editing factors CRR4, CRR21 and CLB19.

Figure S7. Alignment of RpO \( B \) sequences to show conservation of the amino acid affected by editing.

Table S1. Gene-specific primers used for amplifying RNA gel blot probes.

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References


