A Phosphofructokinase B-Type Carbohydrate Kinase Family Protein, NARA5, for Massive Expressions of Plastid-Encoded Photosynthetic Genes in Arabidopsis

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To date, there have been no reports on screening for mutants defective in the massive accumulation of Rubisco in higher plants. Here, we describe a screening method based on the toxic accumulation of ammonia in the presence of methionine sulfoximine, a specific inhibitor of glutamine synthetase, during photorespiration initiated by the oxygenase reaction of Rubisco in Arabidopsis (Arabidopsis thaliana). Five recessive mutants with decreased amounts of Rubisco were identified and designated as nara mutants, as they contained a mutation in genes necessary for the achievement of Rubisco accumulation. The nara5-1 mutant showed markedly lower levels of plastid-encoded photosynthetic proteins, including Rubisco. Map-based cloning revealed that NARA5 encoded a chloroplast phosphofructokinase B-type carbohydrate kinase family protein of unknown function. The NARA5 protein fused to green fluorescent protein localized in chloroplasts. We conducted expression analyses of photosynthetic genes during light-induced greening of etiolated seedlings of nara5-1 and the T-DNA insertion mutant, nara5-2. Our results strongly suggest that NARA5 is indispensable for hyperexpression of photosynthetic genes encoded in the plastid genome, particularly rbcL.

Plastids are DNA-containing organelles that show several types of morphology and functions depending on environmental stimuli and tissue specificity (López-Juez and Pyke, 2005). Chloroplasts are one type of plastid that accumulate massive amounts of proteins that are essential for photosynthesis. The enzyme responsible for photosynthetic CO₂ fixation is Rubisco. This enzyme accounts for 20% to 35% of total leaf nitrogen in higher plants and is the most abundant protein on earth (Ellis, 1979; Evans, 1989).

The complex transcriptional, translational, and folding-assembly machineries in chloroplasts, therefore, have evolved to support this massive accumulation of photosynthetic proteins (Mullet, 1993; Mayfield et al., 1995; López-Juez and Pyke, 2005).

Cyanobacteria have approximately 3,000 to 7,000 genes in their genomes and produce sufficient proteins for cells to adapt to their environment. The chloroplast genome contains only about 100 genes, and the genes for the remaining 3,000 chloroplast proteins are located in the chromosomal DNA (Leister, 2003; López-Juez and Pyke, 2005). This has necessitated the cell to evolve systems that coordinate the expression of the chloroplast genes and their complementary chromosomal genes. In recent years, biochemical and genetic studies have identified various important chromosomal genes involved in biogenesis of chloroplasts (Goldschmidt-Clermont, 1998; Barkan and Goldschmidt-Clermont, 2000; Leister and Schneider, 2003). In particular, genetic analyses have strongly contributed to the construction of a generalized model in which many chromosomal genes are involved in individual processes of gene expression in chloroplasts. Mutants of model plants have been screened on a large scale, based on visible phenotypes such as growth retardation, abnormal leaf color, and physiological abnormality. These analyses have identified various factors involved in either global or individual gene expression in chloroplasts.
The concentration of the Rubisco protein in the stroma of chloroplasts is approximately 200 mg mL^{-1} (Yokota and Shigeoka, 2008), and this is entirely synthesized during leaf development (Irving and Robinson, 2006). In higher plants, Rubisco comprises eight large and eight small subunits (LSU and SSU, respectively), encoded by rbcL in the chloroplast genome and RbcS in the chromosomal DNA, respectively. Many factors participate in the processes, such as transcription of rbcL and RbcS (Manzara and Gruissem, 1988; Tyagi and Gaur, 2003; Shiina et al., 2005), post-transcriptional regulation and translation of rbcL (Barkan and Goldschmidt-Clermont, 2000; Zerges, 2000; Marin-Navarro et al., 2007), transport of SSU precursors through chloroplast envelopes (Kessler and Schnell, 2006), posttranslational modification of LSU and SSU (Houtz and Portis, 2003; Houtz et al., 2008), and folding and association of these subunits in the stroma (Roy and Andrews, 2000). Most of these factors are not specific to Rubisco synthesis but are selectively or universally involved in the synthesis of other chloroplast proteins. For example, the plastid-encoded plastid RNA polymerase (PEP) mainly transcribes rbcL and other photosynthetic genes encoded in the chloroplast genome with the aid of nucleus-encoded sigma factors (Shiina et al., 2005). Ribosome release factor (APG3) is required to synthesize chloroplast proteins (including Rubisco) whose mRNAs contain UAA/UAG stop codons (Motohashi et al., 2007). The Toc complexes such as Toc33 and Toc159 are critical to selectively import photosynthetic precursor proteins into the chloroplast, whereas Tic110 is a component shared by many photosynthetic and nonphotosynthetic precursor proteins (Kessler and Schnell, 2006). The only factor specifically required for successful folding of LSU in both maize (Zea mays) and tobacco (Nicotiana tabacum) is Bundle Sheath Defective2 (BSD2; Brutnell et al., 1999; Wostrikoff and Stern, 2007). These factors were identified through biochemical and reverse, partly forward, genetic analyses based on visible phenotypes or chlorophyll fluorescence.

To understand the coordinated synthesis of Rubisco and other photosynthetic proteins in chloroplasts, we can use new screening techniques to isolate mutant plants. To our knowledge, screening for mutants that are defective in the large-scale accumulation of Rubisco has never been reported. Here, we describe a method to screen for Arabidopsis (Arabidopsis thaliana) expression mutants of plastid-encoded photosynthetic genes.

**Figure 1.** Schematic illustration of the nara screening method. A, Effect of MSX on photorespiratory carbon and nitrogen metabolism. 1, Rubisco; 2, AGT; 3, GS. B, Method for isolating nara mutants. EMS-mutagenized seedlings, wild type (negative control) and rca (positive control), were first grown on selective plates in the presence of 5% CO2 at low light (LL) intensity (prescreening conditions) for 2 to 3 weeks. Plates were then transferred to ambient air at high light (HL) intensity to screen for mutants. After 100 to 150 h, MSX-resistant mutants and rca survive these conditions. Surviving plants were grown on selection medium without MSX in 5% CO2 for 2 to 3 weeks before being transplanted into soil and grown until self-pollination. Using M3 progeny, these screening steps were repeated at least twice more to obtain stable mutants. PG, 2-Phosphoglycolate; PGA, 3-phosphoglycerate; RuBP, ribulose-1,5-bisP.
mutants that cannot accumulate high concentrations of Rubisco due to a mutation in a gene necessary for the achievement of Rubisco accumulation (nara). The method relies on the toxic functions of NH₃ that accumulate in the presence of Met sulfoximine (MSX), a specific inhibitor of Gln synthetase (GS), during photorespiration initiated by the oxygenase reaction of Rubisco. Of the five mutants with decreased amounts of Rubisco, we selected the mutant with the lowest amount of Rubisco in chloroplasts for detailed analyses. This mutant was defective in the function of a phosphofructokinase B (pfkB)-type carbohydrate kinase family protein that is indispensable for hyperexpression of photosynthetic genes encoded in the plastid genome.

Figure 2. Arabidopsis with high Rubisco activity was killed by MSX treatment as a result of ammonia accumulation. A, Visible phenotype of Arabidopsis under screening conditions. Wild-type (WT) and rca plants were grown on selection medium with and without MSX in 5% CO₂ for 2 weeks (left) and then were kept in screening conditions for 100 h (right). Bars = 5 mm. B, Accumulation of ammonium ion in shoots under screening conditions. Wild-type and rca plants were harvested at 0, 20, and 50 h of screening conditions. Ammonium in extracts of plants was assayed according to the method of De Block et al. (1987). Data represent means ± so of three independent samples, with two to five shoots each. FW, Fresh weight; HH, high light; LL, low light.

Figure 3. Screening of nara mutants in the M3 generation. Plants were kept for 120 to 150 h under screening conditions. Only nara10 mutants were isolated by growing plants in prescreening conditions in the presence of 0.3% CO₂. WT, Wild type. Bars = 3 mm.
RESULTS

Evaluation of the Screening Method

It has been estimated that Rubisco catalyzes the oxygenase reaction in photosynthesizing Arabidopsis leaves at 13 μmol g⁻¹ fresh weight h⁻¹ (Sweetlove et al., 2006). This corresponds to the release of NH₃ at 6.5 μmol g⁻¹ fresh weight h⁻¹ at the Gly decarboxylation step during photorespiration. This amount of NH₃ far exceeds amounts released from other metabolic pathways in C₃ plant leaves (Keys et al., 1978; Frantz et al., 1982). Photorespiratory NH₃ is mainly reassimilated by GS₂ in chloroplasts (Fig. 1A). The herbicide MSX kills plants by inhibiting the GS reaction (Lea and Ridley, 1989). Its harmful effects on plant growth result from blocking of Gln synthesis and accumulation of toxic NH₃. In this screening method, the selection medium contained Gln as the sole nitrogen source; thus, the inhibitory effect of MSX on plant growth is restricted to the accumulation of photorespiratory NH₃ (Fig. 1A). Accordingly, mutants with lower levels of Rubisco or strongly deactivated Rubisco in the absence of Rubisco activase are expected to survive in ambient air conditions because of their slower photorespiration rate. The wild-type plants would survive in a CO₂-enriched atmosphere because photorespiration is suppressed (Fig. 1B).

The wild type and the rca mutant of Arabidopsis were grown in air supplemented with 5% CO₂ at 50 μmol photons m⁻² s⁻¹ for 2 weeks. The medium contained 30 mM Gln with or without 50 μM MSX (Fig. 2A). The plants were then transferred to ambient air and grown at 130 to 190 μmol photons m⁻² s⁻¹. The rca mutant was used as the positive control in our screening experiments. This mutant lacks Rubisco activase, which promotes carbamylation of the Rubisco active sites (Somerville et al., 1982; Orozco et al., 1993). There was no visible effect of MSX on the wild type and rca in the presence of 5% CO₂ at both the low and high light intensities (Fig. 2A, left; Supplemental Fig. S1A). After wild-type plants were transferred to ambient air conditions with high light intensity, plants grown on MSX-containing medium died within 100 h (Fig. 2A, right). At low light intensity, however, the wild-type plants grown in ambient air conditions grew, although slowly (Supplemental Fig. S1B). The inhibitory effects of MSX on wild-type plants indicate that the Rubisco oxygenase reaction has a role in the observed growth inhibition. Consistent with this, the growth of the rca mutant, which has little Rubisco activity in air (Salvucci et al., 1986), was not visibly affected by MSX. These results suggest that MSX-resistant Arabidopsis mutants screened under photorespiratory conditions have lower activities of the oxygenase reaction of Rubisco.

To examine the inhibitory effect of MSX on GS activity under these screening conditions, we measured the amount of NH₄⁺ in plants grown in the presence of 50 μM MSX under photorespiratory conditions (Fig. 2B). In wild-type plants, the level of NH₄⁺ was constantly low at approximately 4 μmol g⁻¹ fresh weight in the presence of 5% CO₂ at 50 μmol photons m⁻² s⁻¹. In wild-type plants, this level increased to approximately 210 μmol g⁻¹ fresh weight at illumination levels of 130 to 190 μmol photons m⁻² s⁻¹ in ambient air, but in rca mutants grown under the same conditions, the level increased to only 30 μmol g⁻¹ fresh weight. The rate of NH₃ release from wild-type plants was 4 to 5 μmol g⁻¹ fresh weight h⁻¹. The absence of MSX did not cause any increase in NH₃ in these experiments.

Screening of nara Mutants with Decreased Levels of Rubisco Protein

Sterilized M2 seeds (approximately 20,000) of ethylmethanesulphonate (EMS)-treated Arabidopsis were germinated on agar plates to screen for mutants with lower levels of Rubisco. Seedlings were grown for 14 d under nonphotorespiratory conditions, then were grown under screening conditions for 100 to 150 h to select for mutants that survived the photorespiratory...
conditions (Figs. 1B and 3). The frequency of MSX-resistant plants was two to three per 1,000 seeds, but many failed to grow or were sterile after transplanting into soil. We collected M3 seeds of 12 MSX-resistant mutants and assessed repeatability of resistance (Fig. 3). Five of the selected mutants showed the recessive phenotype. We measured fresh weight of aerial parts, Rubisco activity, and amount of Rubisco protein in 14-d-old mutant lines. Compared with the wild type, the mutant phenotype appeared in one-third of plants of nara5, nara7, and nara10, and nara3 in F2 progeny (Table I). This result indicates that phenotypic suppression in lines 3, 7, and 10. Although rca had a lower activation state of Rubisco, this was not the case in the mutant lines selected in this study. The observed lower initial and total activities of Rubisco were due to decreased amounts of the Rubisco protein in these mutants (Fig. 4B). These mutants were designated as nara mutants.

To examine genetic dominance and genetic segregation of the mutated genes, these mutants were crossed with the wild-type Landsberg erecta. Plants of all F1 progeny showed no detectable growth phenotype, but the mutant phenotype appeared in one-third of plants of nara3 to nara5, nara7, and nara10 in F2 progeny (Table I). This result indicates that phenotypes in these mutants are due to recessive mutations at single genetic loci.

Map-Based Cloning of Genetic Loci in the nara Mutants

We identified genetic loci by map-based cloning of the mutations in the nara3, -5, -7, and -10 mutants. We found a T-to-C substitution at +2,047 of the At5g16180 coding sequence in nara3 (Table II; Supplemental Fig. S2). At5g16180 encodes Chloroplast RNA Splicing1 (AtCRS1), which participates in splicing of the intron in atpF and protein translation in chloroplasts (Till et al., 2001; Asakura and Barkan, 2006). This point mutation changed the codon for Gln at position 683 into a stop codon. Other mutations of this gene have been reported previously: a T-DNA insertion into the first exon of the genomic AtCrs1-1 results in an albino phenotype, and a T-DNA insertion in the sixth exon of the another allele, AtCrs1-2, results in variegated leaves (Asakura and Barkan, 2006). The leaves of the nara3 mutant showed less variegation compared with those of AtCrs1-2 (Fig. 3). This nonsense mutation in the nara3 mutant suggests that deletion of the C-terminal region containing 37 amino acids of AtCRS1 (720AA) weakens the function of AtCRS1. It is likely that the decreased amounts of Rubisco in the nara3 mutant are due to inhibition of chloroplast translation.

The nara5 mutant showed a severely impaired growth phenotype with a very low level of Rubisco. Map-based cloning showed that the genetic locus of NARA5 was in a 120-kb-long genomic sequence located between two polymorphic markers on the two bacterial artificial chromosome clones, M4I22 and T27E11, on chromosome IV. Sequencing of genes with annotations and plastid-leading sequences in this region revealed a G-to-A substitution at +626 of the At4g27600 coding sequence (Fig. 5A; Supplemental Fig. S2). This substitution changes Gly at the 209th residue to Glu (Fig. 5, A and B). We introduced the full-length cDNA of At4g27600 under the control of the cauliflower mosaic virus 35S promoter into the nara5 mutant. This resulted in complete recoveries in appearance and Rubisco content to those of the wild type (Fig. 6, A and C). Thus, we concluded that the phenotype observed in the nara5 mutant was due to the point mutation in At4g27600. At4g27600 encodes a 471-amino acid pfk-type carbohydrate kinase family protein with unknown function, as described later. This newly found protein was named NARA5.

Map-based cloning of the nara7 mutant identified a G-to-A substitution at +1,156 of the At2g13360 coding sequence (Table II). At2g13360 encodes alanine:glyoxylate aminotransferase1 (AGT1), which catalyzes the aminotransferase reaction between Ser and glyox-

| Name | Mutated Protein | Substitution of Nucleotide and Amino Acid | Gene Function of Wild-Type Alleles | Localization |
|------|----------------|------------------------------------------|----------------------------------|--------------|
| nara3 | Chloroplast RNA splicing1 | CAA → TAA<br>Gln-683 → stop codon | Intron splicing of chloroplast atpF and chloroplast translation | Chloroplasts* |
| nara7 | Alanine:glyoxylate aminotransferase | GCC → ACC<br>Ala-386 → Thr | Photorespiratory metabolism | Peroxisomes |
| nara10 | Glutamyl-tRNA synthetase | TCT → TTT<br>Ser-310 → Phe | Synthesis of Glu-tRNA | Chloroplasts<br>Mitochondria |

*TargetP predicted that AtCRS1 was localized in chloroplasts.
Expression Mutants of Plastid-Encoded Photosynthetic Genes

Figure 5. (Legend appears on following page.)
ylate in the photorespiratory pathway (Fig. 1A). Growth inhibition of the nara7 mutant was completely removed by 0.3% CO₂ as observed previously in an AGT1-deficient mutant (Somerville and Somerville, 1980; Liepman and Olsen, 2001). The mutation in At2g13360 caused AtAGT1 to have Thr in place of a highly conserved Ala at the 386th residue. The similar severe phenotypes of the nara7 mutant and AGT1-deficient mutants (Supplemental Fig. S3) suggest that Ala-386 has a pervasive influence on AGT1 activity.

The nara10 mutant had a C-to-T point mutation at +929 in At5g64050, which encodes glutamyl-tRNA synthetase (GluRS; Table II). This mutation substituted Phe for Ser at the 310th residue, which is highly conserved among GluRS enzymes of other organisms. The GluRS is essential for protein synthesis in both chloroplasts and mitochondria and for chlorophyll synthesis in chloroplasts (Duchene et al., 2005). A null mutant of this gene in Arabidopsis shows an ovule abortion phenotype (Berg et al., 2005).

Of all the nara mutants, nara5 showed the greatest decrease in Rubisco content; therefore, it was selected for further detailed analyses and characterization.
Figure 6. Phenotypes of plants with nara5 alleles and chloroplast localization of NARAS-sGFP. A, Phenotypes of nara5 mutants and complemented lines. Plants were grown on Murashige and Skoog medium for 14 d. Bars = 0.5 cm. B, Expression level of NARA5 mRNAs isolated from shoots of 14-d-old wild-type (WT) and nara5 plants were used to analyze NARA5 expression. Act8 was amplified at the same time and was used as the loading control. C, SDS-PAGE for analysis of the Rubisco content in nara5 expression mutants of plastid-encoded photosynthetic genes. D, Chlorophyll, GFP, and Merge images for WT and Pro35S::NARA5 sGFP/nara5-1.
in rosette leaves, with 20% lower levels in the cauline leaves and inflorescence tissues (Supplemental Fig. S5). NARA5 mRNA levels were lowest in the stems. This expression pattern is comparable to that of the Rubisco gene transcripts in higher plants (Manzara and Gruissem, 1988).

The long N-terminal nonconserved sequence (+1 to +117) of NARA5 and its function proposed in this study suggest that NARA5 is localized in chloroplasts (Fig. 5B). Indeed, TargetP predicted that the N-terminal sequence was a chloroplast transit sequence. To analyze the localization of NARA5, we introduced a binary sequence containing the sGFP gene fused to the C terminus of the full-length NARA5 sequence under the control of the cauliflower mosaic virus 35S promoter into plants with the nara5 alleles. The NARA5-sGFP fusion protein completely complemented the phenotype and the Rubisco content in the mutants (Fig. 6A). NARA5-sGFP signals were visualized as green spots clearly superimposed on the red fluorescence from protochlorophyllide and chlorophyll (Fig. 7). These results suggest that NARA5 is localized in chloroplasts.

**Phenotype Analysis of nara5 Mutants**

We compared chlorophyll fluorescence parameters between the wild type and nara5-1 (Table III). Compared with the wild type, the maximum quantum yield (yield) and the quantum efficiency of PSII (Fv/Fm) were severely reduced in the nara5-1 mutant, and the reduction level of the primary electron-accepting plastoquinone of PSII (1 − qP), was increased. These results suggest that, in addition to Rubisco accumulation, photochemical activities are also impaired in nara5-1.

Western blotting of chloroplast proteins encoded by the chloroplast and chromosomal genomes in both alleles of nara5 is shown in Figure 6. Compared with the wild type, the levels of D1, D2, cytochrome (Cyt) b6, and PsAa, as well as LSU of Rubisco, all encoded by the plastid genome, were reduced by approximately 80% in nara5-1 and by up to 90% in nara5-2. Such severe reductions in levels of photosynthetic proteins other than Rubisco were not observed in the rbcL-deficient mutant of tobacco (Allahverdiyeva et al., 2005). In addition to these plastid-encoded proteins, the nucleus-encoded γ-subunit of ATP synthase showed similarly reduced levels. In protein complexes such as ATP synthase, which include subunits encoded by both nuclear and plastid genomes, the expression of the γ-subunit gene in nuclei may have been affected by expression of the partner gene in chloroplasts. On the other hand, expression of the nucleus-encoded plastid proteins Fru-bisP aldolase (FBA) and ferredoxin-NADP+ oxidoreductase (FNR) was unaffected in these mutants. These results suggest that NARA5 is involved in the synthesis and/or maintenance of proteins coded by the plastid genome.

**Aberrant Expression of Plastid-Encoded Photosynthetic Genes during Greening in nara5 Mutants**

During greening of leaves, large amounts of photosynthetic proteins including Rubisco are synthesized in a relatively short time (Sasaki et al., 1985; Mayfield et al., 1995). We examined the accumulation of photosynthetic proteins during leaf greening of nara5 mutants (Fig. 8). Accumulation of the Rubisco LSU in etioplasts was strongly repressed during growth of nara5 mutants in the dark (see time 0). Illumination of dark-grown wild-type seedlings caused massive accumulation of plastid-encoded LSU, D2, and Cyt b6 and nucleus-encoded FBA and FNR. On the contrary, nara5 mutants could not massively accumulate plastid-encoded proteins during greening, while the increase in nucleus-encoded proteins was unaffected.

To determine the mechanism by which expression of plastid-encoded photosynthetic genes is repressed in nara5 mutants, we measured transcript levels of these genes in dark-grown and greening nara5 mutants using real-time PCR. Since these photosynthetic genes are transcribed by PEP, we also measured transcripts of clpP, a plastid-encoded gene transcribed by PEP and nucleus-encoded RNA polymerase (NEP), and rpoB transcribed only by NEP (Fig. 8B). Expression data are relative values and are compared with the level in dark-grown wild-type plants at time 0. The expression of genes transcribed by PEP was more severely suppressed in nara5 mutants than those transcribed by NEP/PEP and NEP. Among the genes examined, the transcript level of rbcL was most severely suppressed in nara5 alleles. The transcript level of the nucleus-encoded RbcS in nara5 mutants was lowered after 10 h of illumination compared with the wild type. This may have been caused by the suppression of plastid gene transcription in the mutant, as has been reported (Rapp and Mullet, 1991). The severe suppression of rbcL transcription may have been a major cause of the decreased Rubisco accumulation in nara5 mutants.

Next, we examined the effect of greening on expression of the NARA5 gene (Fig. 8C). A basal level of NARA5 transcripts was found in etiolated cotyledons, but this level increased 4.5-fold during greening under illumination for 60 h.
Together, these results indicate that NARA5 has a role in expression of PEP-transcribed photosynthetic genes, particularly \( rbcL \).

**DISCUSSION**

Here, we describe a new method to screen for Arabidopsis mutants with decreased Rubisco activity (Fig. 1). We identified mutants with low Rubisco activity by selecting plants that were resistant to the inhibitory effects of MSX on photorespiratory metabolism. Wild-type Arabidopsis plants are killed by MSX as a result of excessive \( \text{NH}_3 \) accumulation from photorespiration, which relies on the Rubisco oxygenase activity. The rate of accumulation of \( \text{NH}_3 \) by the wild type in the presence of MSX (Fig. 2B) was similar to that reported for glycolate metabolism in Arabidopsis (Sweetlove et al., 2006). Both \( \text{NH}_3 \) and its ionized form \( \text{NH}_4^+ \) are harmful to plant growth (van der Eerden, 1982; Britto and Kronzucker, 2002). The \( rca \) mutant survived in the presence of MSX (Fig. 2), because this mutant has lower Rubisco activity in vivo (Fig. 4), as reported previously (Salvucci et al., 1986; Eckardt et al., 1997). On the other hand, wild-type plants were resistant to MSX when grown with higher concentrations of \( \text{CO}_2 \) (i.e. when the Rubisco oxygenase reaction is blocked by competitive inhibition of \( \text{CO}_2 \); Fig. 2A). The five mutants screened in this study had lower levels of Rubisco protein compared with wild-type Arabidopsis (Fig. 4). These results indicate that our positive screening method can identify mutants defective in various factors associated with activity or biosynthesis of Rubisco.

The reduced Rubisco levels in the \( nara3\), -4, -5, -7, and -10 mutants examined were accompanied by impaired growth phenotypes and yellow/pale green foliage (Fig. 3). Mutants with similar phenotypes can occur without influence on Rubisco content. For example, Rubisco levels in the chaperonin 60β-subunit and Hsp100-deficient mutants were relatively unchanged (Ishikawa et al., 2003; Sjögren et al., 2004). In contrast, the nature of the chromosomal mutations in the \( nara3\), -5, and -10 lines indicates mutations to genes whose products are necessary for the biosynthesis of chloroplast proteins, including Rubisco (Table II). The \( nara7 \) mutant had a mutation in the gene for AGT1, which functions in photorespiratory metabolism, but the main reason for the low-Rubisco phenotype in this mutant remains unclear.

Among the \( nara \) mutants, \( nara5 \) showed the most severely defective phenotype for accumulation of plastid-encoded photosynthetic proteins, including Rubisco (Figs. 4, 6, and 8). Many mutants of higher plants that show severely decreased amounts of photosynthetic proteins are defective in factors involved directly in the biosynthetic process. Mutants with defects in genes encoding BSD2, Toc159, and APG3, which participate in either translational or posttranslational processes in chloroplasts, show albino or yellowish phenotypes and accumulate very low levels of Rubisco (Brutnell et al., 1999; Bauer et al., 2000; Motohashi et al., 2007). Mutations in \( nara5 \) alleles resulted in similar phenotypes to these mutants and resulted in very low Rubisco content. These results

### Table III. Photosynthetic parameters of the wild type and \( nara5-1 \)

Data represent means ± se (\( n=3 \)).

| Plant   | Chlorophyll a Fluorescence Parameters | \( F/F' \_\_m \) | \( \Phi PSII \) | \( 1-qP \) |
|---------|--------------------------------------|-----------------|----------------|------------|
| Wild type | 0.81 ± 0.01         | 0.61 ± 0.01     | 0.18 ± 0.01    |
| \( nara5-1 \) | 0.42 ± 0.07         | 0.13 ± 0.03     | 0.70 ± 0.10    |

Figure 7. Quantities of plastid-encoded photosynthetic proteins in \( nara5 \) mutants. A, Immunodetection of plastid-encoded chloroplast proteins with anti-Rubisco LSU, anti-D1, anti-D2, anti-Cyt \( b_6 \), and anti-PsaA antibodies. WT, Wild type. B, Immunodetection of nucleus-encoded photosynthetic proteins with anti-chloroplast ATP γ-subunit, anti-FNR, and anti-chloroplast FBA antibodies and a mitochondrial protein with anti-COXII in \( nara5 \) mutants. Total protein was extracted from shoots of 2- and 3-week-old seedlings for \( nara5-1 \) and \( nara5-2 \) mutants, respectively. The protein amount loaded was 20 μg each for the WT1, \( nara5-1 \), WT, and \( nara5-2 \) lanes. The protein concentration in the wild type was diluted as indicated.
suggest that NARA5 is involved in a process closely related to Rubisco biosynthesis in chloroplasts.

The pfkB family of proteins contains many carbohydrate kinases, such as RK, ADK, GSK, fructokinase, tagatose-6-P kinase, and others (Bork et al., 1993). NARA5 showed no significant homology to these pfkB-type carbohydrate kinases, except for two motifs that are conserved in the pfkB family (Fig. 5B). NARA5 clustered with proteins with similar sequences in a separate clade from RK, ADK, and GSK (Fig. 5C). The recombinant NARA5 protein did not show any of these enzyme activities.

Although the catalytic function of NARA5 is unclear, our data suggest two possible roles for this
protein in influencing expression of chloroplast genes. First, NARA5 may be involved in the PEP-dependent transcription of plastid-encoded photosynthetic genes. In support of this, two pfkB-type carbohydrate kinase family proteins coded by At1g69200 and At1g54090 were identified together with PEP subunits in a transcriptionally active chromosome complex from Arabidopsis and mustard (Sinapis alba) chloroplasts (Pfalz et al., 2006). Also, a tobacco homolog of At1g54090 was detected in an affinity-purified PEP complex from tobacco chloroplasts (Suzuki et al. 2004). These results suggest that the pfkB-type carbohydrate kinase family proteins may take part in PEP-dependent transcription. The PEP transcription system functions in the active transcription of plastid-encoded photosynthetic genes such as rbcL and other photosynthetic genes and also in transcription of rpl16 and some tRNAs (Allison et al., 1996; Hajdukiewicz et al., 1997; Kanamaru et al., 2001; Ishizaki et al., 2005). The facts that NARA5 is a member of the pfkB-type carbohydrate kinase family (Fig. 5) and that transcript levels of genes transcribed by PEP were decreased in the nara5 mutant (Fig. 8B) suggest that NARA5 plays a role in transcription of plastid-encoded genes by PEP. Furthermore, the transcript level of rbcL was the most severely decreased among all of the PEP-transcribed plastid genes (Fig. 8B), which suggests that NARA5 participates in transcription of rbcL rather than other PEP-transcribed genes. In Arabidopsis, the specificity of the PEP transcription system is modulated by six nucleus-encoded sigma factors. Interestingly, however, this preferential reduction in the transcript level of rbcL has not been observed in any sigma factor-null mutant (Kanamaru et al., 2001; Tsunoyama et al., 2004; Favory et al., 2005; Ishizaki et al., 2005; Zghidi et al., 2006; Tozawa et al., 2007). Although there has been no report of a protein factor that is involved in the preferential transcription of rbcL, there might be a mechanism by which NARA5 strengthens preferential transcription of rbcL.

The second possibility is that NARA5 may stabilize transcripts of photosynthetic genes, mainly rbcL. Stabilization of mRNA is an important factor in defining transcript levels in chloroplasts. It has been known that trans-acting factors bind to cis-sequences in the target mRNAs and protect them from degradation by endoribonucleases and exoribonucleases (Herrin and Nickelsen, 2004; Bollenbach et al., 2007), for example, in the pentatricopeptide repeat proteins in the transcriptionally active chromosome complex (Pfalz et al., 2006). Taken together, these findings suggest that NARA5 may combine with these proteins to stabilize mRNA, particularly that of rbcL.

In summary, NARA5 is a pfkB-type carbohydrate kinase family protein that is indispensable for massive accumulation of plastid-encoded photosynthetic protein. Our findings suggest that it participates in the transcription and/or posttranscriptional steps of photosynthetic gene expression such as rbcL in chloroplasts. Future studies will also continue to analyze these proteins and screen for additional Rubisco-deficient nara mutants in a bid to identify key proteins that regulate its synthesis (and that of other proteins) in chloroplasts to expand our understanding of this complex process.

**Materials and Methods**

**Plant Materials**

We used Arabidopsis (Arabidopsis thaliana ecotype Columbia [Col-0]) as the wild-type, the mutant lines rcs (Somerville et al., 1982) and nara, and the GABI-KAT line 718C05 (nara-2). All nara mutants were back-crossed with wild-type Col-0 at least three times except nara-5-2, which was back-crossed twice. The plants were grown on Murashige and Skoog medium supplemented with 3% (w/v) Suc, 0.5 g L\(^{-1}\) MES (pH 5.7), 0.8 μM nicotinic acid, 0.4 μM pyridoxine hydrochloride, 2.4 μM thiamine hydrochloride, 550 μM myoinositol, and 0.8% (w/v) agar, with a 16-h-light/8-h-dark cycle at 23°C, unless otherwise specified.

**Mutant Screening**

We screened nara mutants by uniformly spreading 40 to 50 seeds of EMS-mutagenized M2 plants in the Col-0 genetic background (Lehle Seeds) on selective plates. The selection medium was NH\(_4\)NO\(_3\)- and KNO\(_3\)-free Murashige and Skoog medium supplemented with 50 μM MSX, 30 μM Cl\(_2\), 3% (w/v) Suc, 19 μM KC\(_1\), 0.5 g L\(^{-1}\) MES (pH 5.7), 0.8 μM nicotinic acid, 0.4 μM pyridoxine hydrochloride, 2.4 μM thiamine hydrochloride, 550 μM myoinositol, and 0.8% (w/v) agar. Plants were initially grown for 2 to 3 weeks in the presence of 5% CO\(_2\) under a 20-h photoperiod (50 μmol photons m\(^{-2}\) s\(^{-1}\))/4 h of dark at 23°C. Plates were then transferred to continuous irradiation at 130 to 190 μmol photons m\(^{-2}\) s\(^{-1}\) in ambient air. After 5 to 6 d, MSX-resistant mutants with green or pale green leaves were selected. Plants were then grown on selection medium without MSX for 2 to 3 weeks and then on soil (Metro-Mix 350; Scott-Sierra Horticultural Products) until self-fertilization, both in 5% CO\(_2\).

**Map-Based Cloning**

Homozygous nara mutants were crossed with Landsberg erecta ecotypes. The F1 plants were self-pollinated, and the resulting F2 plants were used for segregation analyses and mapping of nara mutations. Genomic DNA was isolated according to Edwards et al. (1991). Mapping was carried out using extracted genomic DNA from 571 F2 progeny for nara3, 557 F2 progeny for nara5, 789 F2 progeny for nara7, and 964 F2 progeny for nara10. The mutations were mapped with molecular markers based on simple sequence length polymorphism (Bell and Ecker, 1994) and cleaved-amplified polymorphic sequence (Konieczny and Ausubel, 1993) markers. Genomic sequences of candidate mutated genes were amplified by PCR using ExTaq DNA polymerase (TaKaRa), and the PCR products were directly sequenced using a BigDye terminator version 3.1 Cycle Sequencing Kit and an ABI PRISM 3100 sequencer.

**DNA Constructs and Plant Transformation**

For expression of NAR5, NAR5-sGFP, and NAR5-His in plants with nara5 alleles, a series of Gateway binary vectors (pGWBs) were used as described by Nakagawa et al. (2007). We obtained the full-length cDNA of NAR5 from cDNA of 14-d-old Arabidopsis seedlings by PCR using the primer pair 5'-AAAAACGAGCTCTAGGCCTTGCATGCTGGTTCCTCCCTCATCCTC-3' (NAR5 forward)/5'-AGAAGATCCGGTCTCCGAGGTCTATCAAGACCCAA-CATCCT-3'. For fusion of sGFP or His to NAR5 at the C terminus, NAR5 cDNAs lacking the stop codon were amplified by PCR using the primer pair...
NARAS forward/5'-AGAGACTGCTGTAGACCCAACATGTGTGCAGA-CAC-3'. Recombination reactions (BP reactions) were used to clone cDNA of NARAS into pDONRR221. Each NARAS cDNA was subsequently introduced into pGWB2, pGWB5, or pGWB8 by recombination reactions (LR reactions) according to the manufacturer’s protocol (Invitrogen). These binary vectors were introduced into Agrobacterium tumefaciens MP90 by electroporation and then into plants with nara5 alleles by the floral dip method.

Rubisco Levels and Activities

Shoots were homogenized in liquid nitrogen with a mortar and pestle and then dissolved in extraction buffer (50 mM HEPES-KOH [pH 8.0], 1 mM EDTA, 1 mM dithiothreitol [DTT], 1 mM MgCl2, and 2 mM phenylmethylsulfonyl fluoride). Rubisco activity was spectrophotometrically assayed by measuring the rate of NADH oxidation in the presence of phosphoglycerate kinase and glyceraldehyde 3-P dehydrogenase (Sharkey et al., 1991) with some modifications. The homogenate was centrifuged at 18,800g for 10 s at 4°C, and aliquots of the supernatant were immediately used for assay for 30 s to determine the initial activity of Rubisco. The reaction mixture contained 50 mM Tris-HCl ([pH 8.0], 1 mM EDTA, 5 mM DTT, 20 mM MgCl2, 20 mM NaHCO3, 2 mM ribulose 1,5-bisP, 0.2 mM NADH, 1 mM ATP, 5 mM creatine phosphate, 10 units of creatine kinase, 40 units of 3-phosphoglycerate kinase, and 40 units of glyceraldehyde 3-P dehydrogenase. Rubisco in the crude extract was activated by 20 mM MgCl2, and 20 mM NaHCO3. After activation for 10 min at room temperature, Rubisco was added to the reaction mixture to determine total activity.

To determine Rubisco content, shoots were homogenized on ice with a mortar and pestle and dissolved in extraction buffer (50 mM HEPES-KOH [pH 8.0], 1 mM EDTA, 1 mM DTT, and 1 mM MgCl2) supplemented with complete protease inhibitor cocktail tablets (Roche). The homogenate was then centrifuged at 18,800g for 20 min at 4°C. Total proteins in the supernatant were separated by native-PAGE on a 3% to 10% gradient gel (PAGEL NPG-310L; Bio-Rad Laboratories). The membrane was immunoreacted with anti-Rubisco LSU, anti-chloroplast FBA, anti-D1, anti-D2, anti-Cyt b6f, anti-γ-subunit of ATP synthase, and anti-FNR antibodies. Antibodies against the chloroplast proteins D1, D2, PsaA, and Cyt b6f were purchased from Agrisera (http://www.agrisera.com/). Immunoreactive proteins were detected using the ECL-Plus Kit (Amersham Biosciences).

RT-PCR

Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen). We treated RNA with FPLC-pure DNase I (Amerham Pharmacia Biotech) for 30 min at 37°C, and then RNA was purified by phenol/chloroform extraction and ethanol precipitation. Single-strand cDNA was synthesized from 0.5 μg of total RNA using an oligo(dT) primer and ReverTraAce (Toyobo), except that cDNA of plastid-encoded genes was synthesized using a random primer (Toyobo) in place of the oligo(dT) primer. Semiquantitative reverse transcription (RT)-PCR was performed using the following specific primers for NARAS: 5'-TGACCGATCTTTGGAGATGA-3' and 5'-CAAGATCACGTCCCTCAT-TACC-3' for Rh5; 5'-CCGCCTCAGTGTCAAGAAAGG-3'/5'-GCCCTTGAGG- CAAATGAACTGA-3'; for pet5, 5'-AGCTCTAGTGTTAGCACAATCAG-3'/5'-AGGACCAAGAATACTTGTCCATTAC-3'. Amplion size and specificity for each primer pair were confirmed by gel electrophoresis. Quantitative RT-PCR of psbA was conducted with primers described by Loschelder et al. (2006). Primers for psbA, psaA, cp47, and rbcL were described by Ankele et al. (2007). Primers for Act2 were as described by Abdel-Ghany and Pilon (2008).

Chlorophyll Fluorescence Analysis

Chlorophyll fluorescence parameters were measured with a MINI-PAM portable chlorophyll fluorometer (Walz) as described elsewhere (Munekage et al., 2002) with some modifications. Plants were dark-acclimated for 10 min before measurements. We used a light pulse of 3,000 μmol photons m⁻² s⁻¹ for 800 ms and actinic light of 120 μmol photons m⁻² s⁻¹ for 5 min to determine the maximal fluorescence and the steady-state fluorescence, respectively.

Microscopic Analysis

NARA5-sGFP fluorescence was observed with an LSM510 confocal laser scanning microscope (Carl Zeiss Microimaging). An argon laser was used as the excitation light source (488 nm), and sGFP signal was detected at an emission band of 500 to 530 nm. Chlorophyll autofluorescence was observed using an LP 650 optical filter.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Conditional lethal effect of MSX on Arabidopsis wild-type plants.

Supplemental Figure S2. Detection of nara3, nara5, nara7, and nara10 mutations using cleaved-amplicon polymorphic sequence markers.

Supplemental Figure S3. Lethal photosreptory phenotype of nara7.

Supplemental Figure S4. Allelism between nara5-1 and nara5-2.

Supplemental Figure S5. Expression levels of NARAS in different tissues of wild-type Arabidopsis.

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