A Young Seedling Stripe2 phenotype in rice is caused by mutation of a chloroplast-localized nucleoside diphosphate kinase 2 required for chloroplast biogenesis

Kunpeng Zhou1, Jiafa Xia1, Yuanlei Wang1, Tingchen Ma1 and Zefu Li1

1Key laboratory of Rice Genetics and Breeding, Rice Research Institute, Anhui Academy of Agricultural Sciences, Hefei, P.R. China

Abstract

Chloroplast development and chlorophyll (Chl) biosynthesis in plants are regulated by many genes, but the underlying molecular mechanisms remain largely elusive. We isolated a rice mutant named yss2 (young seedling stripe2) with a striated seedling phenotype beginning from leaf 2 of delayed plant growth. The mutant developed normal green leaves from leaf 5, but reduced tillering and chlorotic leaves and panicles appeared later. Chlorotic yss2 seedlings have decreased pigment contents and impaired chloroplast development. Genetic analysis showed that the mutant phenotype was due to a single recessive gene. Positional cloning and sequence analysis identified a single nucleotide substitution in YSS2 gene causing an amino acid change from Gly to Asp. The YSS2 allele encodes a mutant phenotype was due to a single recessive gene. Positional cloning and sequence analysis identified a single nucleotide substitution in YSS2 gene causing an amino acid change from Gly to Asp. The YSS2 allele encodes a mutant phenotype was due to a single recessive gene. Positional cloning and sequence analysis identified a single nucleotide substitution in YSS2 gene causing an amino acid change from Gly to Asp. The YSS2 allele encodes a mutant phenotype was due to a single recessive gene. Positional cloning and sequence analysis identified a single nucleotide substitution in YSS2 gene causing an amino acid change from Gly to Asp. The YSS2 allele encodes a mutant phenotype was due to a single recessive gene. Positional cloning and sequence analysis identified a single nucleotide substitution in YSS2 gene causing an amino acid change from Gly to Asp. The YSS2 allele encodes a mutant phenotype was due to a single recessive gene. Positional cloning and sequence analysis identified a single nucleotide substitution in YSS2 gene causing an amino acid change from Gly to Asp. The YSS2 allele encodes a mutant phenotype was due to a single recessive gene. Positional cloning and sequence analysis identified a single nucleotide substitution in YSS2 gene causing an amino acid change from Gly to Asp. The YSS2 allele encodes a mutant phenotype was due to a single recessive gene. Positional cloning and sequence analysis identified a single nucleotide substitution in YSS2 gene causing an amino acid change from Gly to Asp. The YSS2 allele encodes a mutant phenotype was due to a single recessive gene. Positional cloning and sequence analysis identified a single nucleotide substitution in YSS2 gene causing an amino acid change from Gly to Asp. The YSS2 allele encodes a mutant phenotype was due to a single recessive gene. Positional cloning and sequence analysis identified a single nucleotide substitution in YSS2 gene caus

Keywords: Chloroplast biogenesis, NDPK, Oryza sativa, positional cloning, YSS2 gene.

Received: October 12, 2016; Accepted: March 30, 2017.

Introduction

Chloroplasts are essential organelles in higher plants. The formation of a mature chloroplast from a proplastid during plant development involves many steps: first, the development of the chloroplast itself followed by the development of a functional photosynthetic apparatus (Mullet, 1993; Sakamoto et al., 2008). There are about 3,000 nuclear-encoded and nearly 120 plastid-encoded chloroplast proteins in higher plants (Timmis et al., 2004; Reumann et al., 2005; Pfälz and Pfannschmidt, 2013). These proteins play important roles in chloroplast development, photosynthesis and plastid transcription (Sakamoto et al., 2008; Dong et al., 2013; Pfälz and Pfannschmidt, 2013; Zhou et al., 2013, 2017). Chloroplast biogenesis from proplastids to mature chloroplast goes through three steps (Kusumi et al., 2010): first, plastid DNA synthesis and plastid division, second, establishment of the plastid transcription/translation apparatus, a key to chloroplast formation, and third, activation of the photosynthetic apparatus. At the molecular level, transcript accumulations from both nuclear and plastid genes are necessary for all three steps (Kusumi et al., 2010). FtsZ (encoding a component of the plastid division machinery) is required for the first step (TerBush et al., 2013); RpoT, rpoA and rpoB, separately encoding NEP, and PEP α and β subunits, respectively, are highly expressed during the second step (Hricová et al., 2006; Steiner et al., 2011; Börner et al., 2015), and rbcS (encoding the small subunit of ribulose-1,5-bisphosphate carboxylase), rbcL (encoding the large subunit of ribulose-1,5-bisphosphate carboxylase) and psbA (encoding the D1 subunit of the PSII complex) are abundant in the third step, which functions in activation of the photosynthetic apparatus (Hwang and Tabita, 1989; Nelson and Yocum, 2006). Chloroplast development is affected by alterations in expression levels of these genes. Similarly, V1, V2, V3, St1 and VYL transcripts were reported to accumulate highly in the first or second steps of chloroplast differentiation and to be required for chloroplast biogenesis (Sugimoto et al., 2004, 2007; Yoo et al., 2009; Kusumi et al., 2011; Dong et al., 2013).

Chl-deficient mutants are ideal materials to study Chl biosynthesis, chloroplast development, and chloroplast...
**NDPK2 and NDPK3 are separately localized in plastids of plants. NDPK1 is a cytoplasm-associated protein whereas they are mainly function in maintaining the metabolic balance between NTPs and NDPs in cells (Hasunuma et al., 2003).** They also have essential roles in cell growth and division, signal transduction and plant stress response (Zimmermann et al., 1999). Three kinds of NDPKs predominate in higher plants. NDPK1 is a cytoplasm-associated protein whereas NDPK2 and NDPK3 are separately localized in plastids and mitochondria (Bollet et al., 2007; Kihara et al., 2011). Different localizations suggest that NDPKs play important roles in different cell compartments. Previous studies demonstrated that NDPK2 is associated with embryo and seed development and involved in response to external stress (Yano et al., 1995; Nato et al., 1997; Kawasaki et al., 2001). However, there is little reported evidence that NDPK2 participates in chloroplast development and chloroplast biogenesis.

In this study, we aimed to characterize a young seedling stripe mutant, yss2. The mutant showed a striated phenotype from leaf 2 to leaf 4 and a normal leaf phenotype thereafter. Plant growth was also delayed and there were fewer tillers per plant than in the wild type. The chlorotic leaf phenotype is associated with decreased pigment levels and aberrant chloroplasts. At heading stage, the uppermost leaves are slightly chlorotic and immature panicles display a degree of whitening. We showed that the symptoms were caused by a mutation in a single locus that was fine-mapped to a 62.4 kb region in chromosome 12. Sequence analysis demonstrated that a single base mutation had occurred in the gene we named as YSS2 and subsequently showed to encode a nucleoside diphosphate kinase 2 (NDPK2) with high similarity to NDPKs in other species. Expression analysis showed that YSS2 was highly expressed in L4 tissues, the key time of chloroplast biogenesis. Subcellular localization showed that YSS2 is a chloroplast-associated protein. These results implied that YSS2 plays important roles in chloroplast biogenesis.

**Materials and Methods**

**Plant materials and growth conditions**

The white leaf and panicle mutant yss2 was identified in an MNU-mutagenized population of *japonica* cultivar Nongyuan 238. Plants were grown in a growth chamber or paddy fields. Crosses between the yss2 mutant and Nongyuan 238 or Nanjing 11 were separately used for genetic analysis and gene mapping. Seeds of cultivars Nongyuan 238 and Nanjing 11 were obtained from the Chinese National Key Facility for Crop Gene Resources and Genetic Improvement in Beijing.

**Determination of pigment contents**

Chls and Car were assayed spectrophotometrically according to methods described previously (Zhou et al., 2013). Leaf samples were collected from second, third, fourth or fifth leaves at the L4 or L6 growth stages and separately marinated in 95% ethanol for 48 h in darkness. Absorbance of supernatants was measured with a DU 800 UV/Vis Spectrophotometer (Beckman Coulter) at 665, 649 and 470 nm.

**Transmission electron microscopy (TEM)**

Leaf samples were prepared for TEM from green and white sections of third leaves in yss2 mutant and from similar positions of wild type at the fully expanded L3 stage. Transverse sections of leaves were fixed in a solution of 2.5% glutaraldehyde and then incubated in 1% OsO4 overnight at 4 °C. After staining with uranyl acetate, tissues were dehydrated through an ethanol series, and embedded in Spurr’s medium before ultrathin sectioning. Samples were air-dried, stained again and observed with a Hitachi H-7650 transmission electron microscope.
Mapping of YSS2

Following initial mapping of the YSS2 locus between Indel/SSR markers ID12-8 and RM3331 on chromosome 12 using 35 F2 mutant segregants, an additional 1,216 F2 plants with striated leaf phenotype were used for fine-mapping. High-density Indel markers were developed based on sequence differences between cv. Nipponbare (ja-ponica) and 93-11 ( indica). Primer pairs designed with Primer Premier 5.0 are listed in Table 1. Full-length cDNA and genomic DNA of the predicted ORFs in wild type and yss2 mutant were amplified and sequenced.

Quantitative real-time RT-PCR

Table 1 - Primer sequences used in this study.

| Name          | Forward sequence (5'-3') | Reverse sequence (5'-3') |
|---------------|--------------------------|--------------------------|
| ID12-7        | GTGGCTGTTTAGGAGCGGT    | CAACCAAACACGACTGCAAC     |
| ID12-8        | CCTAGTTCAGCTCTCGTACC   | GCAGAAGAGAAGTTGTGTCG     |
| F41-5         | TGTCGAAAAATTGCTGTTG    | CATGGGGGAAAGTGAGGGA      |
| F41-18        | AGAGCGAGTACATAAGGCAAC  | ATCCGAATACCTCGTCAAC      |
| F41-22        | GAAGATTTCTGCTCGCTTT    | TCCATGTCGAACATTAGCA      |
| F41-25        | CGATATCGTTGCTGCTGCA    | CTGGGTCAAATCTCAAATTTAGT  |
| F41-35        | TCTCTCCCTAGGCTCTCTC   | GTTGGCATTAACTCATTATTT    |
| F41-37        | CGGATAGCGAGCTGAGAGTT  | TATGACTTTGACCTTTCGCC     |
| F41-44        | AGCCAAATCCCAAGGAAACAA | CCCACGCTGATAGATG         |
| F41-48        | CAAAAGGGTGATTTATTTATGTC | GGTGCTGCTGCTGCTC         |
| F41-55        | AAAATCGTGGGAGGAAATAAACA | GCGCCGCAATTCATTGCC       |

Primers for quantitative RT-PCR and subcellular localization

| Name         | Forward sequence (5'-3') | Reverse sequence (5'-3') |
|--------------|--------------------------|--------------------------|
| YSS2-RT1     | GCGTCTCGTCTGAGCAAT       | AGTGCTCCTGCTGCAAGTCC     |
| PORA         | ATCACAAGGGCTACCTTCCTC   | GAGTTGTGTGCTCACACTCTCA   |
| HEMA1        | CACCCAGTCTGAATCTAT       | CTACCACCTCTCTTAATCC      |
| YGL1         | TGGAGCTGGAGATGGTT        | GAATAGGACGAGTAGGT        |
| CHLI         | AGTAGGCTGGTAGTG          | AATGCCTCAACATTCACACT     |
| CHLH         | CTATACACTTCACCAACT      | TACACCAACTCCACACT       |
| CHLD         | GGCAAAGAGAGGCAATAGTAG    | CAAATGCATACATAGATG       |
| rbcL         | GTTGAAGGAGTATAGGAGAGA   | AATATGGTGATGATTTATG      |
| rbcS         | TCAACTATCGCTGCTCTC      | ACTGGGAAACACACAAAAAC     |
| psbA         | AAATCGTGGGAGGAAATAGA    | ATAGCCTGAAATGAAAAAGA     |
| LHCP         | TCTCTCCCGTTGCTCTCTC     | GAGCAGGTTTGCTGCTGCTG     |
| psaB         | TTGGATTTGCCATCCACACAT   | CCGACGTCCTGATAAGAGAT     |
| psbB         | TCCATATGCTTGCGGATCAT   | AGTTGTGGACCATACACTCAA    |
| psbC         | TACAAACCTGGCCAAAGCAAGCA | TACGGCCACACAGAATTATTA   |
| FtsZ         | TTGGGTTTTTCTGACGACAA    | CCTCAATAGCAGACCGATG      |
| RpoA         | AAATCTGTATCGGCCACAC    | ATCCACATTGCAAGGCCGCA     |
| RpoB         | GCATTCTGGACAGATATGATT  | GCGGATGGAATATCGGAGTA     |
| Ubq          | GCCTCGCTCGCTGAGCTATC    | CCGGACGTCAGACCTCTAG      |
| YSS2-GFP     | TTCTAGAAATGGACGCCATGGCCTG | CGGGATCTCTCACCAGCCATTG |
Subcellular localization

The coding sequence of YSS2 was amplified and cloned to the N-terminus of GFP in the transient expression vector pA7-GFP (primer pairs shown in Table 1). Fusion plasmid YSS2-GFP and free GFP were separately transformed into rice protoplasts and incubated in darkness at 28 °C for 16 h before examination (Chiu et al., 2000) programs. GFP fluorescence was observed with a confocal laser scanning microscope (Carl Zeiss LSM700).

Results

Phenotypic characterization of the yss2 mutant

The yss2 mutant was originated from an N-methyl-N-nitroso urea (MNU) mutagenized population of japonica cultivar (cv.) Nongyuan 238. The mutant seedlings displayed a striated leaf phenotype in seedling leaves 2 to 4 under paddy field conditions (Figure 1A,B). However, the fifth and later leaves had normal green phenotype (Figure 1C,D). The yss2 mutant showed delayed seedling growth compared to wild type (Figure 1A-D). To further characterize the mutant, we determined the pigment levels of chloroplasts of green sections (basal section) of yss2 leaves using TEM. The chloroplasts of green sections (basal section) of yss2 leaves had well-developed lamellar structures with normally stacked grana and thylakoid membranes similar to wild type plants (Figure 1M,N); however, chloroplasts in the white segments were undifferentiated (Figure 1O,P). Collectively, our data showed that the yss2 mutation caused a chlorotic defect that disrupted chloroplast development and delayed seedling growth.

Cloning of the YSS2 gene

For genetic analysis of the YSS2 locus, reciprocal crosses between yss2 mutant and Nongyuan 238 were made to determine the mode of inheritance of the yss2 phenotype. F1 plants showed the wild type phenotype, and the F2 populations segregated 3 green : 1 stripe (Table 2). Thus the yss2 phenotype was caused by a single recessive nuclear gene.

To investigate the effect of yss2 on chloroplast development we compared the ultrastructures of chloroplasts in yss2 mutant and wild type seedlings using TEM. The chloroplasts of green sections (basal section) of yss2 leaves had well-developed lamellar structures with normally stacked grana and thylakoid membranes similar to wild type plants (Figure 1M,N); however, chloroplasts in the white segments were undifferentiated (Figure 1O,P). Collectively, our data showed that the yss2 mutation caused a chlorotic defect that disrupted chloroplast development and delayed seedling growth.

Table 2 - Segregation of green and striated seedlings in F2 populations from two crosses.

| Cross               | No. green plants | No. stripe plants | \( \chi^2_{3,3} \) |
|--------------------|-----------------|------------------|-----------------|
| yss2/               | 455             | 140              | 0.686           |
| Nongyuan238         |                 |                  |                 |
| yss2/ Nongyuan238   | 336             | 104              | 0.436           |
| Pooled             | 791             | 244              | 0.446           |

*Value for significance at p = 0.05 and 1 df is 3.84
Figure 1 - Phenotypic characteristics of the yss2 mutant. Wild type and yss2 mutant plants at the four-leaf (A, B) and six-leaf (C, D) stages in a paddy field. Bars, 5 cm. Pigment contents of the wild-type and yss2 mutant plants in different leaf sections at the four-leaf (E) and six-leaf (F) stages (e.g., L4-2, L4-3 separately represent the second and third leaves at the L4 growth stage). Values are means ± SD from three independent determinations. (G) Heights of wild type and yss2 mutant at the six-leaf stage. Values are means ± SD from five independent repeats. (H) Phenotypes of wild type and yss2 mutant at the maximum tillering stage. Bar, 10 cm. (I, J) The yss2 mutant exhibiting striated leaves and white panicles after heading. (K, L) Tiller numbers and plant heights of wild type and yss2 mutant after heading. Electron micrographs showing ultrastructures of chloroplasts from leaf 3 of wild type (M) as well as the green (N) and chlorotic sections (O, P) of leaf 3 in the yss2 mutant at the three-leaf stage. Cp, chloroplast; TM, thylakoid membrane.
Figure 2 - Positional cloning of the YSS2 gene. (A) The yss2 locus was mapped to a 5.4 Mb region between Indel/SSR markers ID12-8 and RM3331 on chromosome 12L. (B) The yss2 locus was narrowed to a 62.4 kb interval between Indel markers F41-37 and F41-55 using 1,216 F2 homozygous mutant plants. (C) Ten ORFs were predicted in the region. (D) Schematic of ORF8 structure. ATG and TAA represent the start and stop codons, respectively. Gray boxes indicate 5' and 3' UTR. Black boxes and lines between them separately indicate exons and introns. The NDPK domain is indicated. The yss2 mutant has a single G to D change in the eighth ORF. The mutation site is indicated by a red arrowhead. The single nucleotide change led to a Gly (G) to Asp (D) substitution. (E) Chromatograms from sequencing of wild type and yss2 genomic DNA. Black frame indicates the mutation site.

Table 3 - Gene prediction within the 62.4 kb region delimited by markers.

| Gene         | Orientation | Annotation                               |
|--------------|-------------|------------------------------------------|
| LOC_Os12g36120 | Forward     | Retrotransposon protein, putative         |
| LOC_Os12g36130 | Forward     | Expressed protein                        |
| LOC_Os12g36140 | Reverse     | Retrotransposon protein, putative         |
| LOC_Os12g36150 | Reverse     | Retrotransposon protein, putative         |
| LOC_Os12g36160 | Reverse     | Expressed protein                        |
| LOC_Os12g36170 | Reverse     | HEAT repeat family protein, putative      |
| LOC_Os12g36180 | Reverse     | Auxilin, putative                        |
| LOC_Os12g36194 | Forward     | Nucleoside diphosphate kinase, putative   |
| LOC_Os12g36210 | Reverse     | Inhibitor I family protein, putative      |
| LOC_Os12g36220 | Reverse     | Inhibitor I family protein, putative      |
YSS2 encodes nucleoside diphosphate kinase 2

The YSS2 allele with seven exons and six introns encodes a polypeptide of 220 amino acid residues with a predicted molecular mass of 23.5 kDa (Figure 2D). The predicted structure indicated that the YSS2 protein contained an NDPK domain covering amino acid residues 73–207 (Figure 3). The YSS2 protein exhibited high similarity to NDPK superfamily proteins in other species across a region of nearly 150 amino acids in the C-terminus. Sequence alignment showed that the Gly83 site is highly conserved in NDPK proteins (Figure 3), suggesting that the site has an essential role.

Phylogenetic analysis revealed that YSS2-like proteins broadly exist in many photosynthetic organisms and likely evolved from the cyanobacteria to angiosperms, thereby forming a large subclade, in which orthologs from monocots and dicots are clearly separated (Figure 4). This suggests that YSS2 is an evolutionarily conserved protein evolved from prokaryotic to eukaryotic genomes.

Gene expression analysis

The expression profiles of OsYSS2, OsNDPK1 and OsNDPK3 were predicted using the Rice Expression Profile Database. YSS2 was expressed in various organs at different growth stages with higher levels in leaf sheaths, stems and ovaries. Overall expression levels were lower than for other NDPKs (Figure 5). OsNDPK1 was mostly expressed in flag leaves, leaf sheaths and roots at the vegetative stage and in stems and ovaries at flowering. OsNDPK3 was highly expressed in all tissues at different developmental stages (Figure 5).
To investigate the expression profile of YSS2 during the process of chloroplast biogenesis, we detected the YSS2 transcripts in different sections of wild type seedlings at the L3 stage. The YSS2 accumulated more in L4 tissues than in leaf 3 and shoot base, and the expression levels were gradually increased with the elongation of leaf 4 (Figure 6A,B). The data revealed that YSS2 was highly expressed in the second step of chloroplast biogenesis.

Given the phenotypic difference between the yss2 mutant and wild type seedlings, we compared the expression levels of genes associated with Chl biosynthesis, photosynthesis and chloroplast biogenesis. Expression levels of Chl biosynthesis-related genes, such as \textit{PORA} (encoding NADPH-dependent protochlorophyllide oxidoreductase), \textit{HEMA1} (encoding glutamyl tRNA reductase), \textit{YGL1} (encoding Chl synthetase), \textit{CHLI} and \textit{CHLD} (encoding Mg-chelatase I and D subunits) were clearly decreased in yss2 seedlings compared to wild type. However, there was no difference in expression of \textit{CHLH} (encoding Mg-chelatase H subunit) (Figure 6C). Expression of genes involved in photosynthesis, such as \textit{rbcL} and \textit{rbcS} (encoding large and small subunits of Rubisco), \textit{psbA}, \textit{psbB} and \textit{psbC} (encoding PSII subunits), \textit{LHCP} (encoding PSII-associated light-harvesting chlorophyll protein) and \textit{psaB} (encoding PSI subunit) was distinctly down-regulated in the mutant (Figure 6D). Genes required for the first (\textit{FtsZ}, encoding a component of the plastid division machinery) and second (\textit{RpoTP}, \textit{rpoA} and \textit{rpoB}, separately encoding NEP, and \textit{PEP} \alpha and \beta subunits) steps of chloroplast biosynthesis were up-regulated in the yss2 mutant compared to wild type (Figure 6E). These data suggested that the YSS2 is involved in the regulatory network of Chl biosynthesis and photo-synthesis as well as chloroplast formation.

Subcellular localization of YSS2

TargetP (Emanuelsson et al., 2000) and ChloroP (Emanuelsson et al., 1999) softwares predicted that YSS2
Figure 5 - Expression analysis of YSS2, OsNDPK1 and OsNDPK3 at various growth stages. Data were collected from the rice expression profile database, RiceXPro.
localizes to chloroplasts and contains a chloroplast-targeting signal of 68 amino acid residues. To detect the actual localization of YSS2, free GFP and YSS2-GFP fusion proteins were each transiently expressed in rice protoplasts. Confocal microscopy confirmed that free GFP was dispersed in the cytoplasm (Figure 7A), whereas YSS2-GFP co-localized with Chl autofluorescence and displayed punctate structures (Figure 7B). The data showed that YSS2 is a chloroplast-associated protein.

Discussion

A number of Chl-deficient and chloroplast development-associated mutants were recently identified in rice. Some of them show chlorotic phenotypes in young seedlings or young leaves and later develop normally, such as ysa, ycl1, yss1 and wsl (Su et al., 2012; Zhou et al., 2013, 2017; Tan et al., 2014). However, some mutants (ygl1 and yv1) display the mutant phenotype and delayed plant growth throughout the entire life cycle (Wu et al., 2007; Dong et al., 2013). This contrasted with the am1 mutant that also exhibited a chlorotic leaf phenotype throughout the life cycle but with little effect on plant development (Sheng et al., 2014). The ygl2 or grc1 mutants showed yellow-green seedling leaf phenotypes but gradually reverted to almost normal green leaves from the tillering stage; both mutants showed delayed plant growth (Chen et al., 2013; Li et al., 2013). wp1 and wlp1 mutants both displayed chlorotic leaves accompanied by white panicles. The relatively weak wp1 mutant had a virescent phenotype and developed white panicles (Wang et al., 2016). The wlp1 mutant produced albinoic leaves until the four-leaf stage but became green at L4 and thereafter; a white panicle appeared at heading (Song et al., 2013). The present white leaf and panicle mutant yss2 showed a striated/chlorotic phenotype at L2, L3 and L4, but normal green leaves at leaf 5 and thereafter. The normal leaf phenotype in yss2 persisted until maximum tillering, somewhat like wlp1 mutant, a slight chlorotic leaf phenotype developed along with white panicles (Figure 1H-K). The chlorotic phenotype presented in young seedlings and panicles suggested that YSS2 might play key roles in young tissues. Genes associated with plastid transcription/translation were largely regulated in wlp1 mutant (Song et al., 2013). Similarly, wp1 impaired chloroplast ribosome biogenesis and reduced plastidic protein synthesis (Wang et al., 2016). We observed that yss2 mutant has phenotypes in seedlings and panicles similar to wp1 and wlp1, suggesting that YSS2 might function at the plastid transcription and translation stages, but further studies are needed for confirmation.

YSS2 was mapped to a 62.4 kb interval on chromosome 12L and 10 ORFs were predicted in the region (Figure 2A-C). Genomic sequence analysis revealed that the
only change in the mutant was in the 8th ORF of YSS2 and was a single base mutation causing an amino acid substitution of Gly by Asp at position 83 (Figure 2D,E). YSS2 was highly expressed in the second step of chloroplast biogenesis, indicating that YSS2 might directly participate in chloroplast formation. Subcellular localization showed that YSS2 is a chloroplast-associated protein (Figure 7B). These data implied that the yss2 mutation might hinder chloroplast biogenesis during early leaf and panicle development, leading to the chlorotic phenotype in young seedlings and panicles. However, we cannot rule out the possibility that YSS2 also has important roles in development of other types of plastids. This is supported by the fact that YSS2 is expressed in non-green tissues (Figure 5). Although there was delayed plant growth and reduced height at the seedling stage the eventual plant height at maturity was similar to the wild type (Figure 1G,L), suggesting that YSS2 paralogs sufficiently compensate for YSS2 function at some growth stages. Expression analysis showed that the housekeeping genes (rpoA and rpoB) and photosynthetic genes (such as rbcl, psbA and psaB) were separately up- and down-regulated in yss2 mutant (Figure 6B,C), suggesting that yss2 might decrease PEP activity and suppress plastid transcription. The dramatically elevated levels of rpo genes and decreased expression of photosynthetic genes indicated that the mutants lacked chloroplast ribosomes or reduced plastid DNA contents (Hess et al., 1993; Udy et al., 2012). We also observed that genes involved in Chl biosynthesis and chloroplast biogenesis were differently regulated (Figure 6C-E), implying that YSS2 might have important roles in the regulatory network of both. Nevertheless, the reduced expression of genes for Chl biosynthesis is not strong and could be an indirect effect such as retrograde plastid-to-nucleus signaling that disturbs the expression of nuclear-encoded chloroplast genes (Mochizuki et al., 2001; Nott et al., 2006).

![Figure 7 - Subcellular location of YSS2. (A) Free GFP signals in rice protoplasts. (B) Transient expression of YSS2-GFP fusion proteins in rice protoplasts. GFP: GFP signals of free GFP and YSS2; Bright: bright field; Auto: chlorophyll autofluorescence; Merged: merged image.](gmb-40-3.pdf)
highly similar amino acid sequences in the NDPK domain, implying that NDPK proteins possess similar functions in different cell compartments. This is the first report that a NDPK protein participates in regulation of Chl biosynthesis and chloroplast biogenesis. Further research on the YSS2 protein could provide new insights into understanding how it participates in these functions, as well as in plant growth.

Acknowledgments

This work was supported by grants from the Natural Science Foundation of Anhui Province (1408085MKL62), the Youth Innovation Fund of Anhui Academy of Agricultural Sciences (16B0101), the 863 Program of China (2014AA10A604-17), and the Key Research and Development Program of China (2016YFD0100101-06). We thank Dr. Bing Hu (Nanjing Agricultural University) for assistance with transmission electron microscopy.

References

Bolter B, Sharma R and Soll J (2007) Localisation of Arabidopsis NDPK2 - Revisited. Planta 226:1059-1065.

Börner T, Aleykinova AY, Zubo YO and Kusnetsov VV (2015) Chloroplast RNA polymerases: Role in chloroplast biogenesis. BBA-Bioenergetics 1847:761-769.

Chen H, Cheng ZJ, Ma XD, Wu H, Liu YL, Zhou KN, Chen YL, Ma WW, Bi JC, Zhang X, et al. (2013) A knockdown mutation of YELLOW-GREEN LEAF2 blocks chlorophyll biosynthesis in rice. Plant Cell Rep 32:1855-1867.

Chen S, Tao L, Zeng L, Vega-Sanchez ME, Umemura K and Wang GL (2006) A highly efficient transient protoplast system for analyzing defence gene expression and protein-protein interactions in rice. Mol Plant Pathol 7:417-427.

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H and Sheen J (1996) Engineered GFP as a vital reporter in plants. Curr Biol 6:325-330.

Dong H, Fei GL, Wu CY, Wu FQ, Sun YY, Chen MJ, Ren YL, Zhou KN, Cheng ZJ, Wang JL, et al. (2013) A rice virecent-yellow leaf mutant reveals new insights into the role and assembly of plastid caseinolytic protease in higher plants. Plant Physiol 162:1867-1880.

Dorion S, Matton DP and Rivoal J (2006) Characterization of a cytosolic nucleoside diphosphate kinase associated with cell division and growth in potato. Planta 224:108-124.

Emanuelsson O, Nielsen H and von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci 8:978-984.

Emanuelsson O, Nielsen H, Brunak S and von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol 300:1005-1016.

Hasunuma K, Yabe N, Yoshiada Y, Ogura Y and Hamada T (2003) Putative functions of nucleoside diphosphate kinase in plants and fungi. J Bioenerg Biomembr 35:57-65.

Hess WR, Prombona B, Fieder B, Subramanian AR and Börner T (1993) Chloroplast rps15 and the rpsob/C1/C2 gene cluster are strongly transcribed in ribosome-deficient plastids: Evidence for a functioning non-chloroplast-encoded RNA polymerase. EMBO J 12:563-571.

Hricová A, Quesada V and Micol JL (2006) The SCABRA3 nuclear gene encodes the plastid RpoTp RNA polymerase, which is required for chloroplast biogenesis and mesophyll cell proliferation in Arabidopsis. Plant Physiol 141:942-956.

Hwang SR and Tabita FR (1989) Cloning and expression of the chloroplast-encoded rbcL and rbcS genes from the marine diatom Cylindrotheca sp. strain N1. Plant Mol Biol 13:69-79.

Kawasaki S, Borchert C, Deyholos M, Wang H, Brazzille S, Kawai K, Galbraith D and Bohnert HJ (2001) Gene expression profiles during the initial phase of salt stress in rice. Plant Cell 13:889-905.

Kihara A, Saburi W, Wakuta S, Kim M-H, Hamada S, Ito H, Imai R and Matsui H (2011) Physiological and biochemical characterization of three nucleoside diphosphate kinase isoforms in rice (Oryza sativa L.) Biochim Biophys Acta 75:1740-1745.

Kusumi K, Chono Y, Shimada H, Gotoh E, Tsuyama M and Iba K (2010) Chloroplast biogenesis during the early stage of leaf development in rice. Plant Biotechnol 27:85-90.

Kusumi K, Sakata C, Nakamura T, Kawasaki S, Yoshimura A and Iba K (2011) A plastid protein NUS1 is essential for build-up of the genetic system for early chloroplast development under cold stress conditions. Plant J 68:1039-1050.

Li JQ, Wang YH, Chai JT, Wang LH, Wang CM, Long WH, Wang D, Wang YH, Zheng M, Poo C, et al. (2013) Green-reversible chlorina 1 (gcr1) is required for the biosynthesis of chlorophyll and the early development of chloroplasts in rice. J Plant Biol 56:326-335.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^-Delta Delta CT method. Methods 25:402-408.

Mochizuki N, Brusslan JA, Larkin R, Nagatani A and Chory J (2001) Arabidopsis genomes uncoupled 5 (GUNC) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction. Proc Natl Acad Sci U S A 98:2053-2058.

Moon H, Lee B, Choi G, Shin D, Prasad T, Lee O, Kwak S-S, Kim DH, Nam J, Bahk J, et al. (2003) NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. Proc Natl Acad Sci U S A 100:358-363.

Mullet JE (1993) Dynamic regulation of chloroplast transcription. Plant Physiol 103:309-313.

Nato A, Mirshahi A, Tichtinsky G, Mirshahi M, Faure JP, Lavergne D, De Buyser J, Jean C, Dureux G and Henry Y (1997) Immunological detection of potential signal-transduction proteins expressed during wheat somatic tissue culture. Plant Physiol 113:801-807.

Nelson N and Yocum CF (2006) Structure and function of photosystems I and II. Annu Rev Plant Biol 57:521-565.

Nott A, Jung HS, Koussevitzky S and Chory J (2006) Plastid-to-nucleus retrograde signaling. Annu Rev Plant Biol 57:739-759.

Pfalz J and Pfannschmidt T (2013) Essential nucleoid proteins in early chloroplast development. Trends Plant Sci 18:186-194.
Reumann S, Inoue K and Keegstra K (2005) Evolution of the general protein import pathway of plastids (review). Mol Membr Biol 22:73-86.

Ryu JS, Kim JI, Kunkel T, Kim BC, Cho DS, Hong SH, Kim S-H, Fernandez AP, Kim Y, Alonso JM, et al. (2005) Phytochrome-specific type 5 phosphatase controls light signal flux by enhancing phytochrome stability and affinity for a signal transducer. Cell 120:395-406.

Sakamoto W, Miyagishima S and Jarvis P (2008) Chloroplast biogenesis: Control of plastid development, protein import, division and inheritance. The Arabidopsis Book 6:e0110.

Sheng PK, Tan JJ, Jin MN, Wu FQ, Zhou KN, Ma WW, Heng YQ, Wang JL, Guo XP, Zhang X, et al. (2014) Albino midrib 1, encoding a putative potassium efflux antiporter, affects chloroplast development and drought tolerance in rice. Plant Cell Rep 33:1581-1594.

Song J, Wei XJ, Shao GN, Sheng ZH, Chen DB, Liu CL, Jiao GA, Xie LH, Tang SQ and Hu PS (2013) The rice nuclear gene \( WLP1 \) encoding a chloroplast ribosome L13 protein is needed for chloroplast development in rice grown under low temperature conditions. Plant Mol Biol 84:301-314.

Steiner S, Schroter Y, Pfalz J and Pfannschmidt T (2011) Identification of essential subunits in the plastid-encoded RNA polymerase complex reveals building blocks for proper plastid development. Plant Physiol 157:1043-1055.

Su N, Hu ML, Wu DX, Wu FQ, Fei GL, Lan Y, Chen XL, Shu XL, Zhang X, Guo XP, et al. (2012) Disruption of a rice pentatricopeptide repeat protein causes a seedling-specific albino phenotype and its utilization to enhance seed purity in hybrid rice production. Plant Physiol 159:227-238.

Sugimoto H, Kusumi K, Tozawa Y, Yazaki J, Kishimoto N, Kikuchi S and Iba K (2004) The \( VIRESCENT-2 \) mutation inhibits translation of plastid transcripts for the plastid genetic system at an early stage of chloroplast differentiation. Plant Cell Physiol 45:985-996.

Sugimoto H, Kusumi K, Noguchi K, Yano M, Yoshimura A and Iba K (2007) The rice nuclear gene, \( VIRESCENT-2 \), is essential for chloroplast development and encodes a novel type of guanylate kinase targeted to plastids and mitochondria. Plant J 52:512-527.

Tan J, Tan Z, Wu F, Sheng P, Heng Y, Wang X, Ren Y, Wang J, Guo X, Zhang X, et al. (2014) A novel chloroplast-localized pentatricopeptide repeat protein involved in splicing affects chloroplast development and abiotic stress response in rice. Mol Plant 7:1329-1349.

TerBush AD, Yoshida Y and Osteryoung KW (2013) FtsZ in chloroplast division: Structure, function and evolution. Curr Opin Cell Biol 25:461-470.

Timmis JN, Ayliffe MA, Huang CY and Martin W (2004) Endosymbiotic gene transfer: Organelle genomes forge eukaryotic chromosomes. Nat Rev Genet 5:123-135.

Udy DB, Belcher S, Williams-Carrier R, Gualberto JM and Barkan A (2012) Effects of reduced chloroplast gene copy number on chloroplast gene expression in maize. Plant Physiol 160:1420-1431.

Wang YL, Wang CM, Zheng M, Lyu J, Xu Y, Li XH, Niu M, Long WH, Wang D, Wang HY, et al. (2016) WHITE PANICLE1, a Val-rRNA synthetase regulating chloroplast ribosome biogenesis in rice, is essential for early chloroplast development. Plant Physiol 170:2120-2123.

Wu Z, Zhang X, He B, Diao L, Sheng S, Wang J, Guo X, Su N, Wang L, Jiang L, et al. (2007) A chlorophyll deficient rice mutant with impaired chlorophyllide esterification in chlorophyll biosynthesis. Plant Physiol 145:29-40.

Yano A, Umeda M and Uchimiya H (1995) Expression of functional proteins of cDNA encoding rice nucleoside diphosphate kinase (NDK) in \( Escherichia coli \) and organ-related alteration of NDK activities during rice seed germination (\( Oryza sativa \)). Plant Mol Biol 27:1053-1058.

Yoo SC, Cho SH, Sugimoto H, Li J, Kusumi K, Koh HJ, Iba K and Paek NC (2009) Rice \( VIRESCENT \) and \( Stripe1 \) encoding the large and small subunits of ribonucleotide reductase are required for chloroplast biogenesis during early leaf development. Plant Physiol 150:388-401.

Zhou KN, Ren YL, Lv J, Wang YH, Liu F, Zhou F, Zhao SL, Chen SH, Peng C, Zhang X, et al. (2013) Young Leaf Chlorosis 1, a chloroplast-localized gene required for chlorophyll and lutein accumulation during early leaf development in rice. Planta 237:279-292.

Zhou KN, Ren YL, Zhou F, Wang Y, Zhang L, Lyu J, Wang YH, Zhao SL, Ma WW, Zhang H, et al. (2017) Young Seedling \( Stripe1 \) encodes a chloroplast nucleoid-associated protein required for chloroplast development in rice seedlings. Planta 245:45-60.

Zimmermann S, Baumann A, Jaekel K, Marbach I, Engelberg D and Frohnmeyer H (1999) UV-responsive genes of \( Arabidopsis \) revealed by similarity to the Gcn4-mediated UV response in yeast. J Biol Chem 274:17017-17024.

Associate Editor: Marcio de Castro Silva Filho

License information: This is an open-access article distributed under the terms of the Creative Commons Attribution License (type CC-BY), which permits unrestricted use, distribution and reproduction in any medium, provided the original article is properly cited.