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ZEBRA2, encoding a carotenoid isomerase, is involved in photoprotection in rice

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Abstract "zebra" mutants have alternating green and chlorotic crossbands on leaf blades and are widely distributed in monocotyledonous crops. Most recently, we cloned the first responsible gene from rice, *ZEBRA2,* which also leads to the phenotype of rice preharvest sprouting. *ZEBRA2*, a single-copy gene in the rice genome, encodes a carotenoid isomerase (CRTISO), the key enzyme catalyzing the conversion of *cis*-lycopene to all-*trans* lycopene. ZEBRA2 shares high identity with known CRTISOs from other species. Expression analysis via both RT-PCR and ZEBRA2-promoter-β-glucuronidase (GUS) transgenic rice indicates that *ZEBRA2* is predominantly expressed in mesophyll cells of mature leaves where active photosynthesis occurs. Consistent with the alteration in agronomic traits, the *zebra2* mutant exhibits decreased photosynthetic rate and chlorophyll content. Mutation of the *ZEBRA2* gene results in the accumulation of all-*trans*-lycopene precursor, prolycopene (7Z,9Z,7′Z,9′Z tetra *cis*-lycopene), in dark-grown *zebra2* tissues. Light-grown *zebra2* mutant exhibits the characteristic "zebra" phenotype and decreased level of lutein, the xanthophyll that is essential for efficient chl triplet quenching. More severe phenotype of the *zebra2* mutant under high light intensity indicates that "zebra" phenotype might be caused by photooxidative damages. We conclude that *ZEBRA2* is involved in photoprotection in rice.

Keywords Carotenoid biosynthesis · Carotenoid isomerase · Rice · *zebra2* mutant

Chenglin Chai, Jun Fang contribute equally to this work.

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Introduction

Carotenoids fulfill an extraordinary variety of functions in plants (Cunningham and Gantt 1998). Besides the roles as membrane stabilizers in chloroplasts and light-harvesting pigments in photosystems, one important role of carotenoids is the protection of the photosynthetic apparatus from damage of reactive oxygen species (ROS) (Bartley and Scolnik 1995; Frank and Cogdell 1996; Havaux 1998; Niyogi 1999). Some carotenoids are precursors for the synthesis of abscisic acid (ABA), an important plant hormone regulating seed maturation, germination, and also adaptation to environmental stresses (Giraudat et al. 1994). As a result, blocking of the carotenoid biosynthesis pathway may result in ABA deficiency, leading to seed-dormancy failure and vivipary or preharvest sprouting (PHS) phenotypes (Fong et al. 1983; Groot and Karssen 1992; Leon-Kloosterziel et al. 1996).

In the carotenoid biosynthesis pathway, carotenoid isomerase (CRTISO) is the key enzyme that converts

cis-lycopene to all-*trans* lycopene via *cis*-configuration intermediates (Breitenbach et al. 2001; Masamoto et al. 2001; Isaacson et al. 2002; Park et al. 2002). Interestingly, the CRTISO activity can partially be substituted by light in green tissues, i.e., photoisomerization (Isaacson et al. 2002; Park et al. 2002). Loss of *CRTISO* causes partial inhibition of lutein biosynthesis in light-grown tissues and appearance of yellow color in newly developed leaves in tomatoes. Also, delayed chlorophyll (chl) accumulation during photomorphogenesis (in an *Arabidopsis CRTISO* mutant *ccr2*) due to the lack of prolamellar body (PLB) has been observed, indicating its role in plastid development and photomorphogenesis (Isaacson et al. 2002; Park et al. 2002; Masamoto et al. 2004). Both *crtiso* mutants exhibit an orange color either in fruit (tomato) or in dark-grown tissue (*Arabidopsis*) due to the accumulation of the all-*trans*-lycopene precursor, prolycopene (7Z, 9Z, 7′Z, 9′Z tetra *cis*-lycopene)(Isaacson et al. 2002; Park et al. 2002). In addition to CRTISO, another isomerase in the carotenoid biosynthesis pathway Z-ISO has been identified in maize and *Arabidopsis*, which catalyzes the step upstream of CRTISO (Janick-Buckner et al. 2001; Li, et al. 2007, Chen et al. 2010). Mutation in *Z-ISO* resulted in conditional accumulation of 9, 15, 9′-tri-*cis*-ζ-carotene (the product of phytoene desaturase, PDS) in dark-grown tissues (Li, et al. 2007; Chen et al. 2010).

"zebra" mutants named by the striking feature of alternating green and yellow (or white) crossbands on leaf blades have been described in a number of monocotyledonous crops such as rice, maize, sorghum, and pearl millet (Coe et al. 1987; Werner and Burton 1991; Oki et al. 1997; He et al. 2000; Kusumi et al. 2000). The appearance of "zebra" phenotype depends to a great extent on environmental factors such as temperature and light, as well as developmental stages (He et al. 2000; Kusumi et al. 2000). The rice "zebra" mutant *1103s* was shown to be thermosensitive and displayed the "zebra" phenotype only after induction by a cold shock in a 'high (28°C)–low (20–23.1°C)–high(26°C or higher)' temperature cycle (He et al. 2000), while another rice "zebra" mutant was dependent on both alternative light/dark growth condition and high light intensity (Kusumi et al. 2000), suggesting that this phenotype is regulated by both photoperiod and light intensity. Moreover, some "zebra" mutants from maize, rice, and pearl millet developed "zebra" phenotype leaves only within early developmental stages followed by a gradually disappearing of this phenotype (Werner and Burton 1991; He et al. 2000; Kusumi et al. 2000). In rice, fourteen non-allelic "zebra" mutants (*zebra1-14*) were identified, and the corresponding genes had been primarily mapped on different chromosomes (http://www.gramene.org/db/genes/search_gene?query=*zebra*&search_field=&species=&gene_type_id=&has_phenotype=&query_submit=Search&order_by=&page_no=1). Although some of these "zebra" mutants have long been used to determine genetic linkages in conventional breeding, the molecular mechanism underlying the phenotype is still enigmatic.

Recently, we reported map-based cloning of the rice *PHS3* gene from our extensive screening of preharvest sprouting mutants (Fang et al. 2008). Interestingly, the *ZEBRA2* was also mapped to the same region and was further confirmed to be *PHS3* by genetic crossing (data not shown). The *zebra2-1* mutant, previously described as *phs3-1*, showed both the PHS and "zebra" phenotypes. In this paper, we provide the detailed data on characterization of *ZEBRA2*, as well as its biologic role in photoprotection.

Materials and methods

Plant materials

All the rice (*Oryza sativa* L. var. *Nipponbare*) plants used in this study were grown in the field unless specified otherwise. For carotenoid analysis, the *zebra2-1* mutant, complemented and RNAi transgenic seedlings, and the corresponding wild-type controls were grown in a growth chamber (either under a 16-h light/8-h dark or under a 24-h dark regime), and shoots of ten-day-old seedlings were harvested. Carotenoids were also extracted from inner parts of mature stems at mature stage and leaves from shaded (approximately 40% of the light intensity of field condition) and non-shaded plants at tillering stage. For chlorophyll analysis, leaves were harvested at tillering stage. For detection of *ZEBRA2* expression in different tissues, total RNA was extracted from shoots and roots of two-week-old seedlings, as well as flag leaves, stems and young panicles of plants at heading stage under natural long-day conditions. For histochemical analysis, flag leaves, roots, internodes, nodes, and flowers (at flowering stage) and immature seeds (at filling stage) were harvested from six *ZEBRA2::*GUS transgenic rice lines.

Pigments analysis and measurement of photosynthetic rate

Carotenoid analysis was carried out according to the reference (Fraser et al. 2000). Measurements were carried out on a HPLC 10AVP system (Shimadzu, http://www.shimadzu.com). A reverse-phase C30 column (250 × 4.6 mm, 5 μm) coupled to a 20 × 4.6 mm C30 guard column (YMC Inc., Wilmington, NC, USA) with the corresponding mobile phases and time programs were used in subsequent HPLC analysis (Fraser et al. 2000). Carotenoids were identified by their characteristic absorption spectra and typical retention time, by comparison with authentic standards and by

referring to literatures (Isaacson et al. 2002; Park et al. 2002). Chl was extracted with 80% acetone and determined as previously described (Arnon 1949). Two independent HPLC runs were carried out. Also, relative abundance of each carotenoid component in the *zebra2-1* mutant (or complemented lines) compared to that in the wild type was obtained by showing the ratio of each peak area (between the *zebra2-1* mutant (or complemented lines) and the wild type) derived from HPLC chromatograms recorded at 430 or 460 nm.

Photosynthetic gas exchange measurements were performed, when the plants grown in the field reached tillering stage, by using a LI-6400 portable photosynthesis system (Li-cor, Lincoln, NE, USA). The parameters were controlled as following: CO_2 concentration 400 μmol s^{-1}, air flow 500 μmol s^{-1}, 28°C and field humidity. A range of light intensities between 0 and 2,500 μmol photons m^{-2} s^{-1} were supplied from a LED source attached to the leaf chamber. Five measurements were performed.

Agronomic trait analysis

Agronomic traits (including plant height, seed setting rate and thousand-grain weight) of both *zebra2-1* mutant and wild type were analyzed at mature stage with 15–20 replicates.

RNA extraction and RT-PCR

Extraction of total RNA (Chomczynski and Sacchi 1987) and synthesis of the first-strand cDNA (Luo et al. 2006) were performed according to previously described methods, respectively. To analyze the gene expression profile for chl, chloroplast biosynthetic and carotenogenic genes, total RNA was extracted from 2-week-old seedlings and tillering stage leaves of wild-type and *zebra2-1* mutant plants, respectively. For RT-PCR analysis, 4 μg of total RNA was digested with DNase I and reverse-transcribed by using the M-MLV Reverse Transcriptase (Promega, USA). Product of the first-strand cDNA synthesis reaction was then used as a template for amplification in a PCR. For semiquantitative PCR, the PCR was run for 28 to 30 cycles at 94°C for 30 s, 55 to 58°C for 30 s and 72°C for 1 min, with an additional extension at 72°C for 8 min. *ACTIN1* gene was used as an internal control for all RT-PCR analysis. PCR products were separated by electrophoresis on 1.2–3.0% (W/V) agarose gel. The quantitative RT-PCR was performed with Bio-Rad CFX96 Real-time System following the protocol as described previously (Tong et al. 2009). Primers used in the PCR were listed in Table S1. Experiments were repeated three times to obtain representative results.

Vector constructions and plant transformation

To create the *ZEBRA2::GUS* construct, the 1,571-bp promoter region of *ZEBRA2* was fused to the *GUS* coding sequence in CAMBIA1301Z (CAMBIA). For the *ZEBRA2* RNAi construct, a 417-bp fragment from 465 to 881 bp of the *ZEBRA2* ORF was inserted as a *Sal*I/*Xba*I fragment in sense orientation downstream of the potato (*Solanum tuberosum* L.) GA20 oxidase intron into pUC-RNAi (Luo et al. 2006). The same fragment was inserted in antisense orientation into the *Spe*I/*Xho*I sites of pUC-RNAi already carrying the sense fragment. Subsequently, the fragment comprising sense and antisense fragments of *ZEBRA2* interspersed by potato GA20 oxidase intron was excised from pUC-RNAi using the flanking *Pst*I and inserted into a pXQAct plasmid between rice *ACTIN1* promoter and Ocs terminator, yielding the binary construct. Construction of complementation and plant transformations for all the constructs were essentially performed as previously described (Liu et al. 2007). The primers used for generating the *ZEBRA2::GUS* and RNAi constructs are listed in Table S1.

Histochemical analysis

Histochemical assay for GUS activity in transgenic plants was performed as described (Jefferson 1989). Various organs of *ZEBRA2::GUS* transgenic rice were incubated in a solution (50 mM sodium phosphate buffer, pH 7.0, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.1% Triton X-100 and 1 mM X-Gluc), directly or after hand-cutting, at 37°C for 12 h followed by incubation in gradient concentrations of ethanol to remove chl.

Phylogenetic analysis

A neighbor-joining tree was built using MEGA version 3.1 (Kumar et al. 2004), adopting Poisson correction distance, and was presented using traditional rectangular TreeView. Support for the tree obtained was assessed using the bootstrap method with 1,000 replicates.

Results

Phenotypes of the *zebra2-1* mutant

Four *zebra2* alleles were originated from different mutations, and *zebra2-1,* previously described as *phs3-1,* which showed a PHS phenotype, was selected for further studies (Fang et al. 2008). Under field conditions, the *zebra2-1*

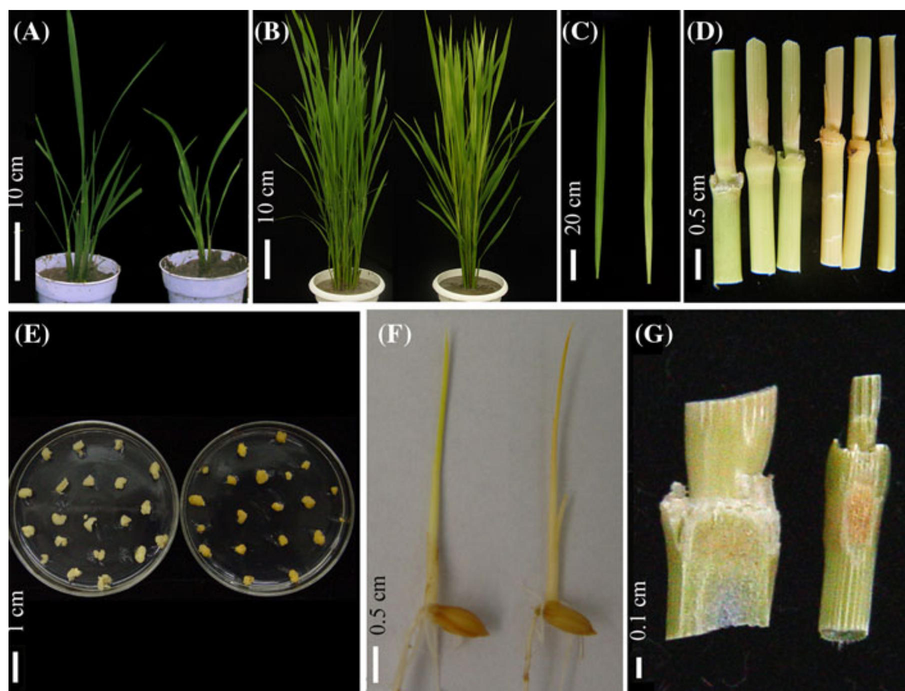

Fig. 1 Morphological characteristics of the *zebra2-1* mutant. (**A**) Phenotypes at early tillering stage (the wild type is on the left and *zebra2-1* mutant right, the same below); (**B**) Phenotypes at late tillering stage; (**C**) Magnified leaves from (**B**); (**D**) Stems at maturation; (**E**) Callus derived from embryos; (**F**) Dark-grown seedlings; (**G**) Inner parts of stems exposed to sunlight

mutant began to exhibit alternating transverse green and yellow sectors on leaves at early tillering stage (Fig. 1A). At late tillering stage, the yellow sectors expanded and turned white, and the newly grown leaves were completely yellow (Fig. 1B, C). In addition, the stem of *zebra2-1* mutant, especially the part around basal nodes at maturation (Fig. 1D), callus derived from *zebra2-1* embryos (Fig. 1E) and the dark-grown *zebra2-1* seedlings showed an orange color (Fig. 1F). Interestingly, the inner part of the stem from the *zebra2-1* mutant also showed an orange color; however, the surface of stem from *zebra2-1* appeared as normally pale green as seen with the wild type (Fig. 1G). The *zebra2-1*mutant also showed altered agronomic traits, including plant height, seed setting rate, and thousand-grain weight (Fig. 2).

The *zebra2-1* mutant shows decreased photosynthetic rate and reduced chl content

To evaluate whether and to what extent the *ZEBRA2* mutation affects photosynthesis, light curves of photosynthetic rates were measured (Fig. 3A). In all light intensities, the photosynthetic rate of yellow leaf sectors of *zebra2-1* was approximately 27–42% of the wild-type leaves, and it reached a maximum at a light intensity of 500 μmol photons m^{-2} s^{-1}, while in the green sectors of *zebra2-1* leaves, the photosynthetic rate was higher than that of the yellow sectors, but still significantly lower than that of the wild type.

For better understanding of the decreased photosynthetic rate of *zebra2-1* mutant, chl content was measured

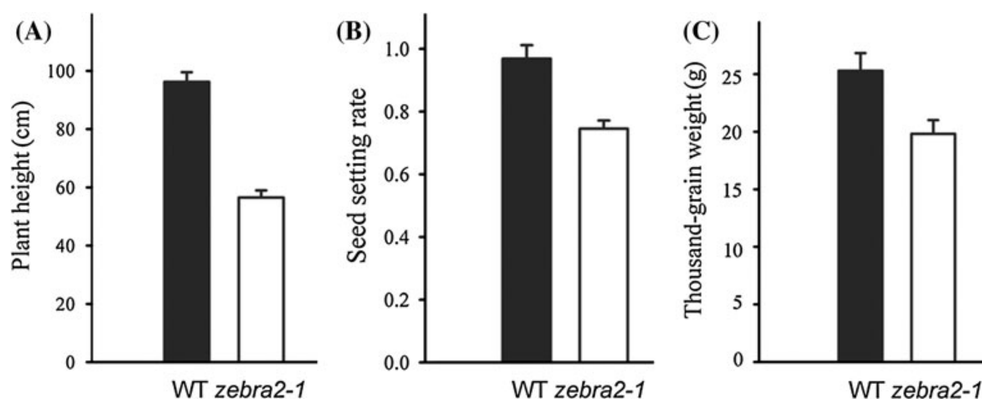

Fig. 2 Agronomic traits of wild type (WT, *black column*) and *zebra2-1* mutant (*blank column*). (**A**) Plant height; (**B**) Seed setting rate, 1.0 represents 100%; (**C**) Thousand-grain weight. Standard deviations were obtained from 15–20 measurements

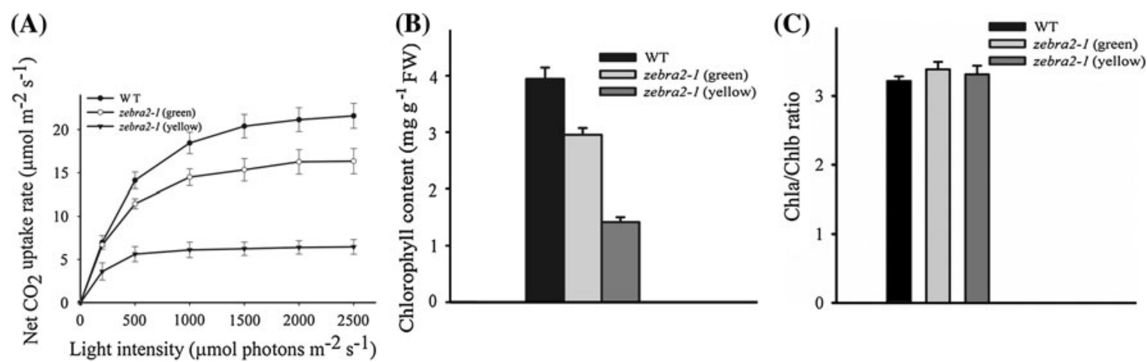

Fig. 3 Photosynthetic rate (**A**), chl content, (**B**) and chl a/chl b ratio (**C**) of wild-type (WT) and *zebra2-1* mutant leaves (including green and yellow tissues). Standard deviations were obtained from seven measurements for (**A**) and from five measurements for (**B**) and (**C**), respectively

(Fig. 3B). The total chl content in green and yellow leaf sectors was approximately 75 and 36% of that in wild-type leaves, respectively. However, the chl a/chl b ratio was not altered (Fig. 3C).

ZEBRA2 encodes an CRTISO in rice

The identity of *ZEBRA2* was confirmed by genetic complementation and RNAi experiments. The preharvest sprouting and “zebra” phenotypes were rescued when the genomic *ZEBRA*2 was introduced, while the RNAi transgenic lines exhibited the “zebra” phenotype (Fang et al. 2008 and Fig. S1). To comprehensively prove that *CRTISO* gene is indeed attributed to the *zebra2-1* phenotype, the expression profiles regarding both size and abundance of *CRTISO* transcript in the *zebra2-1* mutant, complemented and RNAi lines were analyzed by semi-quantitative PCR and quantitative real-time PCR, respectively. The *CRTISO* transcript in *zebra2-1* mutants is 24 bp shorter than that of the wild type due to an alteration in the splicing site, while both of the transcripts were observed in the complemented line, indicating the wild-type *CRTISO* gene has been successfully transformed into the *zebra2-1* mutant and well expressed (Fig. S2). The quantitative real-time PCR was conducted to verify the abundance of CRTISO in those lines accordingly (Fig. S3). The abundance of *CRTISO* transcript in the complemented line is comparable to (or even higher than) that in the wild type, while the transcript in *zebra2-1* mutants and RNAi lines is significantly reduced compared to the wild type (Fig. S3). Taken together, we conclude that the “zebra” phenotype can indeed be attributed to the mutation in *CRTISO/ZEBRA2* gene.

ZEBRA2 is a single-copy gene in the rice genome and predicted to be 1,761 bp long in cDNA sequence (from ATG to TAG) and composed of 13 exons. This gene structure is consistent with *CRTISO*s in tomato and *Arabidopsis* (Isaacson et al. 2002; Park et al. 2002). The *ZEBRA2* ORF encodes a 586-amino acid protein with a 49-amino acid transit peptide at N-terminal, which was predicted to localize the protein to chloroplasts (data not shown).

To determine the evolutionary relationship between ZEBRA2 and CRTISO orthologs from other organisms, an unrooted tree was built using neighbor-joining method based on full-length protein sequences (Fig. 4). Phylogenetic analysis indicated that the CRTISO orthologs were clearly divided into two groups: group I comprised members from higher plants and group II from prokaryotic organisms. The rice ZEBRA2 belongs to group I and shows closer phylogenetic relationship to CRTISOs from grass family including maize and sorghum than to other monocotyledonous species, such as *Oncidium* cv. Gower Ramsey, or dicotyledonous species, indicating its evolutionary process. ZEBRA2 also shares considerable identity (56–59%) with bacterial carotenoid desaturase and FAD-dependent oxidoreductase, suggesting its involvement in similar redox reactions.

Carotenoid biosynthesis was impaired in the *zebra2-1* mutant

Since *ZEBRA2* is a single-copy gene in the rice genome, and if ZEBRA2 is a functional CRTISO in rice, mutations in *ZEBRA2* gene should cause alterations in the carotenoid composition. To test this possibility, HPLC was performed using various tissues, and peaks were identified by their absorbance spectra (Fig. S4). Under light-growing conditions, both the *zebra2-1* and wild-type plants showed very similar carotenoid profiles except for decreased lutein and α-carotene levels, and an increased zeaxanthin level in the *zebra2-1* mutant (Fig. 5A and Table S2). No statistically significant changes for other carotenoid components were likely due to the compensatory photoisomerization of *cis*-carotenoid occurring in light (Isaacson et al. 2002; Park et al. 2002). To avoid the interference from

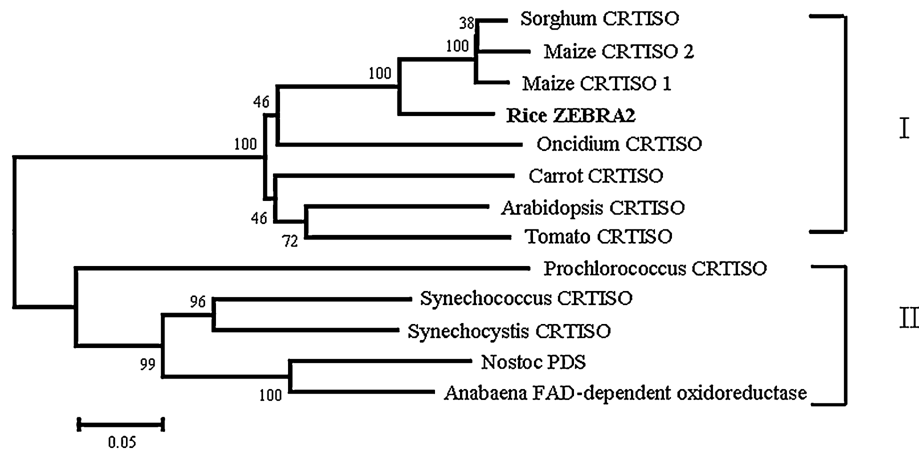

Fig. 4 Phylogenetic analysis of ZEBRA2. Neighbor-joining tree was built on the full-length protein sequences of rice ZEBRA2/CRTISO and other representative CRTISO orthologs from diverse organisms. The *scale bar* is an indicator of genetic distance based on branch length. Group I includes the rice ZEBRA2/CRTISO and CRTISOs from maize (ACO71189 for ZmCRTISO 1 and NP_001148055 for ZmCRTISO 2, respectively), sorghum (XP_002449729), *Arabidopsis* (NP_172167), tomato (Q8S4R4), carrot (*Daucus carota*) (Q2VEX9), and Oncidium (Q52QW3). Group II includes CRTISOs from *Prochlorococcus* (*Prochlorococcus marinus* str. NATL2A) (YP_291830), *Synechococcus* (*Synechococcus elongatus* PCC 6301) (YP_171014) and *Synechocystis* (*Synechocystis* sp. PCC 6803) (NP_442727), *Nostoc* (*Nostoc punctiforme* PCC 73102) PDS (ZP_00108188), and *Anabaena* (*Anabaena variabilis* ATCC 29413) FAD-dependent oxidoreductase (YP_323615)

photoisomerization, we further investigated the carotenoid composition of *zebra2-1* mutant grown in the darkness. In etiolated *zebra2-1* seedlings, the major carotenoids that accumulated were prolycopene (7Z, 9Z, 7′Z, 9′Z tetra *cis*-lycopene), the all-*trans*-lycopene precursor, and carotenoids in *cis*-configuration (Fig. 5B). However, we only detected the accumulation of neoxanthin, violaxanthin, and lutein at low levels in both the wild type and complemented etiolated tissues under 430 nm wavelength (Fig. 5B). Those *cis*-carotenoids are at high level in the *zebra2-1* mutant but at very low level in the wild-type plant, resulting in more than 10,000 times of relative abundance in *zebra2-1* mutant compared to the wild type (Table S3). These results were consistent with reports from two other *CRTISO* mutants, *ccr2*, an *Arabidopsis* mutant that accumulated prolycopene in etiolated seedlings, and *tangerine*, a tomato mutant that accumulated prolycopene as the major carotenoid in fruits (Isaacson et al. 2002; Park et al. 2002).

The complemented transgenic plant and the wild type had comparable carotenoid compositions both in light-grown leaves and in etiolated seedlings (Fig. 5A, B and Table S2, S3), indicating the wild-type genomic *ZEBRA2* can also rescue the *zebra2-1* phenotype regarding the carotenoid composition.

Interestingly, carotenoids extracted from inner parts of the *zebra2-1* stem were very similar to those from etiolated *zebra2-1* seedlings (Fig. 5C and Table S4), which accumulate high levels of prolycopene and its precursors. This suggests that the orange color of these two tissues was caused by high level of carotenoids in *cis*-configuration.

Expression pattern of *ZEBRA2*

To determine the expression pattern of *ZEBRA2*, mRNA level in various tissues from wild-type plants, including young shoots, young roots, stems, young panicles, and mature leaves was detected. *ZEBRA2* is expressed in all tissues/organs tested with its highest expression in mature leaves and relatively low expression in the other tissues (Fig. 6).

To get more expression information of *ZEBRA2*, a binary vector containing the *uidA* gene (*β*-glucuronidase, GUS) driven by the *ZEBRA2* promoter (*ZEBRA2::GUS*) was constructed, and 13 independent (*ZEBRA2::GUS*) transgenic lines were generated by *Agrobacterium*-mediated transformation. Six independent *ZEBRA2::GUS* transgenic lines were used to examine *GUS* expression. Histochemical localization revealed that the GUS expression was exclusively restricted to mesophyll cells in leaves and vascular bundles in roots, respectively (Fig. 7A, B). In immature seeds, *GUS* expression was found in the seed coat and embryos but not in the endosperms (Fig. 7C). However, GUS activities were observed in all tissues of internodes, nodes, and flowers (Fig. 7D–F).

Expression pattern of stress-responsive and carotenogenic genes in the *zebra2-1* mutant

As it has been shown that *PSY3* was up-regulated by abiotic stimuli including salt and drought stresses (Welsch et al. 2008), the normal light condition for wild-type rice growing might be a stress condition for the *zebra2* mutants

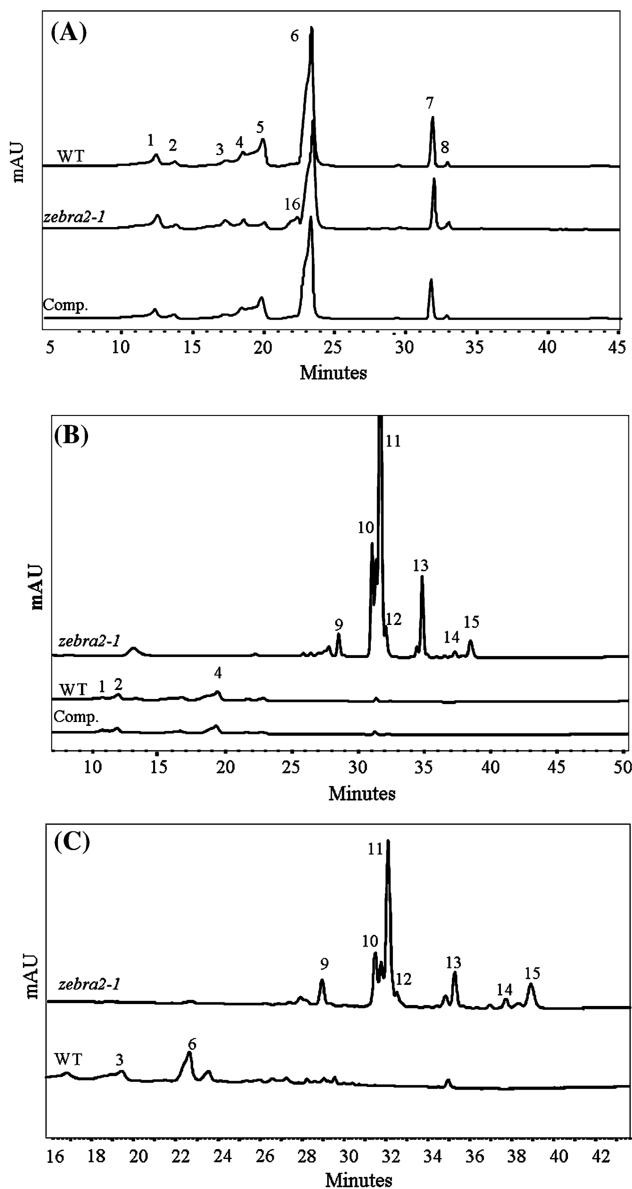

Fig. 5 HPLC chromatograms recorded at 430 nm of light-grown (**A**) and etiolated (**B**) wild-type, *zebra2-1* mutant, and complementation transgenic seedlings (Comp.), as well as (**C**) stems of the wild-type and *zebra2-1* mutant. Peak 1, neoxanthin; peak 2, violaxanthin; peak 3, chl b; peak 4, lutein; peak 5, α-carotene; peak 6, chl a; peak 7, β-carotene isomer 1; peak 8, β-carotene isomer 2; peak 9, ζ-carotene isomer 1; peak 10, ζ-carotene isomer 2; peak 11, prolycopene; peak 12, *cis*-lycopene isomer 1; peak 13, neurosporene isomer 1; peak 14, *cis*-lycopene isomer 2; peak 15, neurosporene isomer 2; peak 16, zeaxanthin

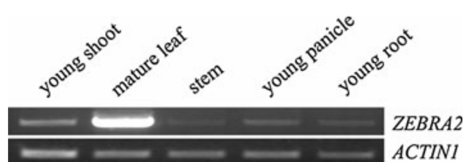

Fig. 6 RT-PCR analysis of the expression patterns of *ZEBRA2*

due to inefficient photoprotective systems (Fang et al. 2008), *PSY3* (encoding phytoene synthase 3) might be up-regulated in the *zebra2-1* mutant. Therefore, we analyzed by quantitative real-time PCR to explore this possibility. Also included in this assay were genes *ZEBRA2*, *PSY1,* and *PSY2* (encoding phytoene synthase 1 and 2, respectively), *PDS* (encoding phytoene desaturase), *ZDS* (encoding zeta-carotene desaturase), *LycB* (encoding lycopene β-cyclase), *LycE* (encoding lycopene ε-cyclase), and *VxDE* (encoding violaxanthin de-epoxidase) to check whether the *ZEBRA2* mutation affects the expression of other carotenogenic genes (Fig. 8A). The transcripts of upstream genes including *PSY1*, *PSY2, PDS, ZEBRA2,* and *LycE* were reduced in the *zebra2-1* mutant compared to those in the wild type. However, none of the transcripts of downstream genes exhibited difference between wild-type and the *zebra2-1* mutant. It is not surprising that there is no significant difference of *PSY3* expression between the wild-type and the *zebra2-1* mutant because *PSY3* was up-regulated by increased ABA level upon salt or drought treatment (Welsch et al. 2008) while the ABA level was decreased in the *zebra2-1* mutant (Fang et al. 2008).

Expression of genes for chl metabolism and chloroplast development in the *zebra2-1* mutant

The chl metabolism and chloroplast development might be disturbed in the *zebra2-1* mutant since it displayed a chlorosis phenotype. We examined the expression of genes for the chl metabolism including *HEMA* (encoding glutamyl-tRNA reductase)*, POR* (encoding NADPH:protochlorophyllide oxidoreductase), *YGL1* (encoding chl synthase), *CAO* (encoding chlorophyllide a oxygenase), *NYC1* (encoding chl b reductase), and those for chloroplast development including *CAB* (encoding the Lhcb1 light-harvesting chl *a/b*-protein of PS II), *RS* (encoding the small subunit of Rubisco), *FZ* (encoding a plastid division protein FtsZ), and *SA* (encoding a sigma factor of a plastid RNA polymerase) (Fig. 8B). Except for the expression of *HEMA* and *POR*, two upstream pathway genes in chl biosynthesis that were slightly increased, expressions of most genes for chl metabolism and chloroplast development were relatively low in the *zebra2-1* mutant compared to the wild type. The disturbance of chl metabolism and chloroplast development in *zebra2-1* mutant might account for the decreased chl content and abnormality in chloroplast development (Fig. 3B and Fang et al. 2008).

The "zebra" phenotype was enhanced by high-intensity light

To further confirm that the "zebra" phenotype was caused by photooxidation, the *zebra2-1* mutant and wild-type

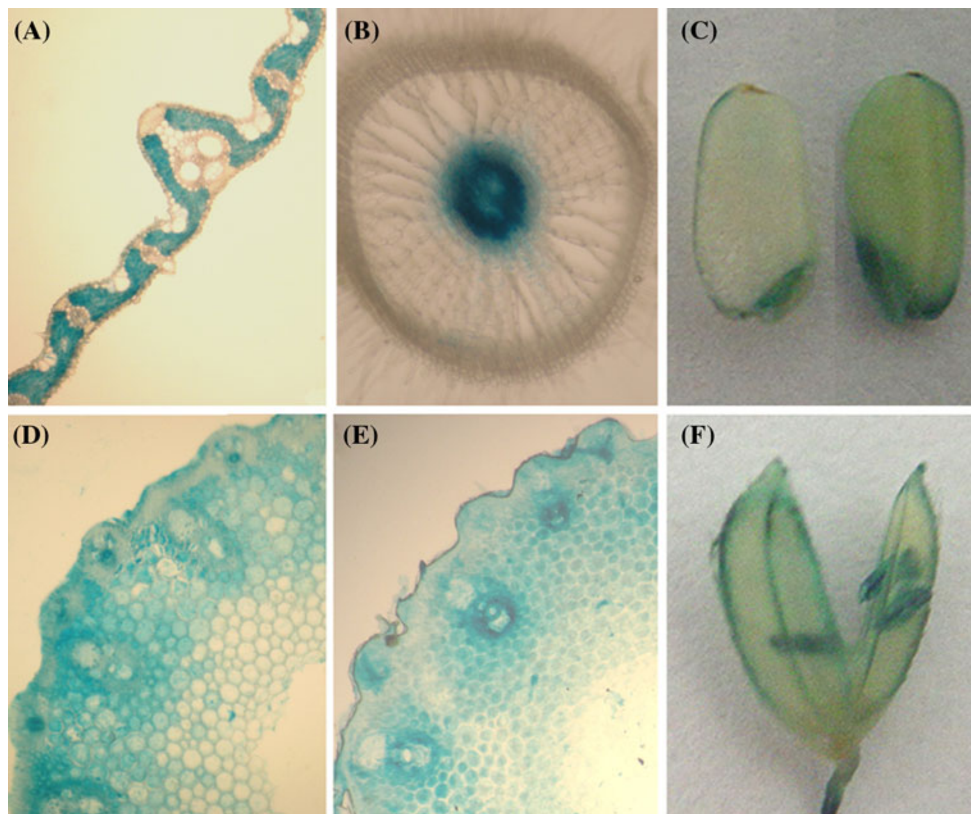

Fig. 7 GUS expression in various tissues in *ProZEBRA2*::GUS transgenic plants. Hand cross-sections of leaf (**A**), root (**B**), seed (**C**), internode (**D**), node (**E**), and flower (**F**)

control plants were grown under different light intensities. Under high light intensity (field condition with the maximum light intensity of 3,000 µmol photons m^{-2} s^{-1}), *zebra2-1* exhibited its characteristic phenotype, while the shaded *zebra2-1* mutant plants (with 40% of the light intensity of field condition) showed a much less severe phenotype (Fig. 9A), indicating that the "zebra" phenotype was enhanced by high-intensity light. Consistent with the chlorosis pattern under different light intensities, the *zebra2-1* plant under non-shaded light condition showed a lower chl and carotenoids level than the shaded one as well as the wild type (Fig. 9B and Table S5). However, the *zebra2-1* mutants had distinguished lower lutein levels than the wild type, regardless of the light intensities. In contrast, the *zebra2-1* mutant exhibited higher zeaxanthin level than the wild type (Fig. 9B and Table S5).

Discussion

"zebra" mutants from different plant species were first reported more than seven decades ago (Hayes 1932; Kadam et al. 1940; Coe et al. 1987; Kusumi et al. 2000), but the molecular mechanisms underlying remain unclear. In rice, fourteen non-allelic "zebra" mutants were identified, and the corresponding genes were mapped on different chromosomes. *ZEBRA2* has been the first gene cloned responsible for the "zebra" phenotype among grasses. The identity of *ZEBRA2* was fully confirmed by transgenic complementation and RNAi experiments, RT-PCR, as well as carotenoid analysis (Fig. 5, Fig. S2, S3 and Table S2, S4).

ZEBRA2 shares high identity with the known CRTISOs, containing the FAD-binding domain and oxidoreductase domain, a feature of other CRTISOs which have been biochemically characterized (Isaacson et al. 2002; Park et al. 2002; Isaacson et al. 2004), indicating the phylogenic conservation in the gene structure. Furthermore, *ZEBRA2* exhibited the conserved gene structure as shown in *CRTISOs* from tomato and *Arabidopsis*, which conferred a similar phenotype (carotenoids profile) when the homologous gene was mutated. Therefore, we conclude *ZEBRA2* is a functional *OsCRTISO* in rice. Unlike other mutants blocked at early steps of the carotenoid biosynthetic pathway, which show lethal albino phenotype (Hable et al. 1998; Conti et al. 2004; Dong et al. 2007; Qin et al. 2007), the *zebra2-1* mutant is variegated and non-lethal. Since CRTISO activity can be partially substituted by light in green tissues (Isaacson et al. 2002; Park et al. 2002), the non-lethal phenotype of the *zebra2-1* mutant may be attributed to the compensatory effects of photoisomerization.

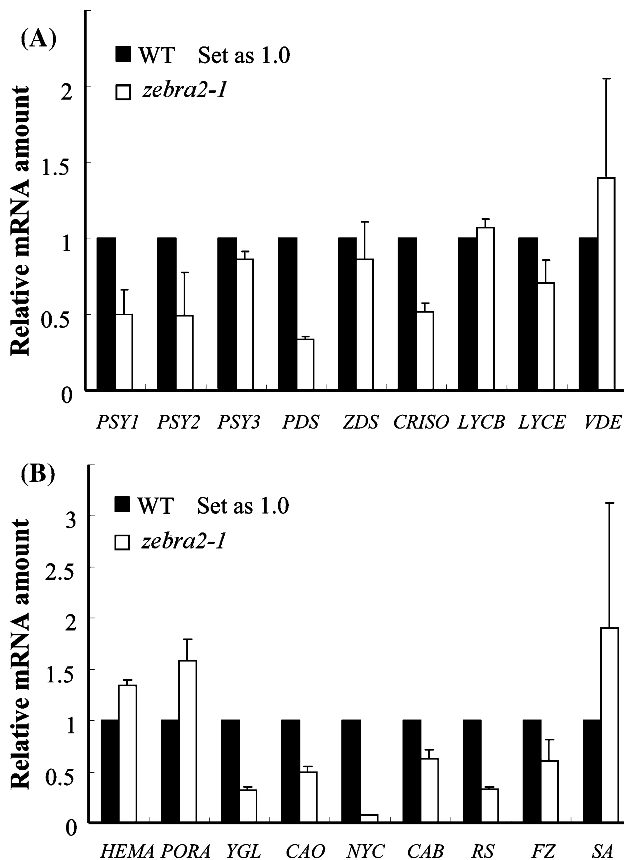

Fig. 8 Expression analyses of some carotenogenic and stress-responsive genes (**A**) and genes of chl and chloroplast development (**B**) in leaves of wild-type (WT) and *zebra2-1* mutant. *ACTIN1* cDNA was used as a control. Reproducible results were obtained from three independent experiments. *PSY*, phytoene synthase; *LycB*, lycopene *β*-cyclase; *LycE,* lycopene ε-cyclase; *PDS*, phytoene desaturase; *VxDE*, violaxanthin de-epoxidase; *ZDS*, ζ-carotene desaturase; *HEMA,* gene encoding glutamyl-tRNA reductase*; POR,* gene encoding NADPH:protochlorophyllide oxidoreductase; *YGL1,* gene encoding chl synthase; *CAO,* gene encoding chlorophyllide a oxygenase; *NYC1,* gene encoding chl b reductase; *CAB;* gene encoding the Lhcb1 light-harvesting chl *a/b*-protein of the PS II; *RS,* gene encoding the small subunit of Rubisco; *FZ,* gene encoding a plastid division protein FtsZ; *SA,* gene encoding a sigma factor of a plastid RNA polymerase

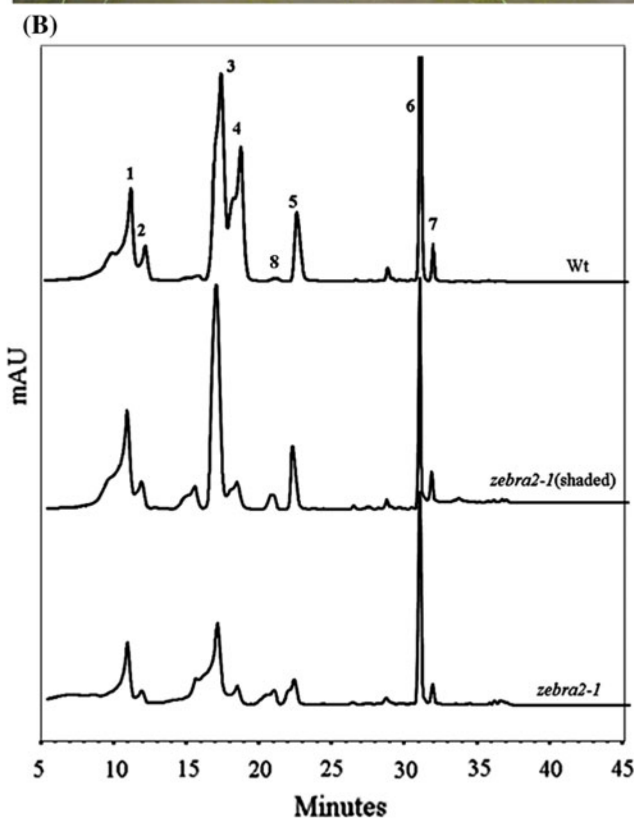

Fig. 9 Phenotypes and carotenoid profiles of wild-type and *zebra2-1* mutant plants under different light intensities. (**A**) phenotypes under field light and shaded conditions (the plants in front were shaded while those behind were not. All the *zebra2-1* mutants were grown on the left and wild type on the right, no matter shaded or not shaded); (**B**) HPLC chromatograms recorded at 460 nm. Peak 1, neoxanthin; peak 2, violaxanthin; peak 3, chl b; peak 4, lutein; peak 5, chl a; peak 6, *β*-carotene isomer 1; peak 7, *β*-carotene isomer 2; peak 8, zeaxanthin

However, CRTISO is not the sole protein responsible for isomerization reactions in the plant carotenoid biosynthesis pathway. A maize gene, *Y9*, encodes a product essential for the *cis*-to-*trans* conversion of the 15-*cis*-bond in 9,15,9′-tri-*cis*-ζ-carotene to 9,9′-di-*cis*-ζ-carotene (Janick-Buckner et al. 2001; Li et al. 2007), the reaction occurring upstream of CRTISO. Both CRTISO and Z-ISO activities can be partially compensated by photoisomerization, which was reflected from the facts of "zebra"/striping phenotype and reduced carotenoids in light-grown tissues from these loss-of-function mutants, respectively (Figs. 5A, B, 9B and Janick-Buckner et al. 2001). The CRTISO was shown to be specific for adjacent *cis*-double bonds at 7, 9 and 7′, 9′ positions, while Z-ISO catalyzes substrates with single *cis*-double bond, suggesting their distinct/complementary roles in carotenoid isomerizations (Janick-Buckner et al. 2001; Isaacson et al. 2002,2004; Park et al. 2002; Li, et al. 2007). Interestingly, *CRTISO* might have evolved from

bacterial *PDS* (*CRTI*), while *Z-ISO* might originate from a bacterial gene *NnrU* that is required for denitrification (Isaacson et al. 2002, 2004; Park et al. 2002; Chen et al. 2010).

The long-standing issue of leaf-variegated mutants, e.g., the *zebra2-1* mutant in this study, is the formation of leaves with green and yellow/white sectors. Cloning and functional analysis of genes responsible for leaf variegation in *Arabidopsis* revealed that it might be caused by a defect in various metabolic pathways related to organelle (mitochondria or chloroplast) functions (Wetzel et al. 1994; Carol et al. 1999; Chen et al. 2000; Takechi et al. 2000; Naested et al. 2004; Rosso et al. 2006). It has been reported repeatedly that an altered carotenoid pool results in loss (or at least partial loss) of photoprotection (Pogson et al. 1998; Niyogi 1999). Lutein is the most abundant carotenoid in photosynthetic apparatus of higher plants and essential for efficient chl triplet quenching (Formaggio et al. 2001; Dall'Osto et al. 2006). However, in agreement with the down-regulation of several carotenogenic genes in the *zebra2-1* mutant (Fig. 8A), the *zebra2-1* mutant shows a dramatically decreased lutein level in light-grown leaves (Figs. 5A, 9B). Possibly due to the lack of this kind of photoprotective pigment, photooxidative damages to the photosynthetic apparatus occurred in *zebra2-1* mutant, and this was also supported by the ROS accumulation, the decreased F_v/F_m and NPQ as well as reduction in the PS II core proteins (Fang et al. 2008). Furthermore, the "zebra" phenotype was enhanced under high light intensity (Fig. 9A) accompanied by decreased chl level (Fig. 9B), indicating that it was caused by photooxidative damages. It was documented that zeaxanthin was accumulated via so-called "xanthophyll cycle" under excess light, and zeaxanthin was proposed to mediate the harmless dissipation of excess energy as heat (Demmig-Adams and Adams 1996). We could also see zeaxanthin accumulation in the *zebra2-1* mutant plant under light condition that is normal for the wild-type but might be a stress for the *zebra2-1* mutant (Fig. 5A and Table S2), and this accumulation in the *zebra2-1* mutant was enhanced when exposed to higher light intensities (Fig. 9B and Table S5). However, the enhanced photooxidative stress under higher light intensity may be not efficiently relieved due to limited protective carotenoid pool in the *zebra2-1* mutant. Taken together, we propose that inactivation of ZEBRA2 mainly causes a dramatic change in carotenoids (especially lutein) content, and the decrease in these photoprotective pigments leads to low efficiency in scavenging ROS in the PS II followed by the accumulation of ROS that results in photoinhibition and photobleaching. However, the mechanism how the segregation of green and yellow tissues occurs precisely is still unknown. The production of variegation may be a common phenotype of interorganellar compensation and cytoplasmic sorting processes (Sakamoto 2003), ensuring that some cells (containing normal chloroplasts) can avoid photooxidative damage and form the green sectors while other cells are photooxidized and form the white sectors (containing defective chloroplasts) (Aluru and Rodermel 2004). In monocotyledonous plants, such as rice, the leaf emerges from the leaf sheath and has a linear gradient of cellular and chloroplast development between leaf base and leaf tip; therefore, the "zebra" phenotypes in rice are thought to be formed in response to the occurrence of periodic changes in some environmental factors (light and/or temperature) during a specific stage of leaf cell development (Kusumi et al. 2000). *CRTISO* may be involved in this series of events, and further study about environmental and developmental factors that influence the expression of *CRTISO* will help us clarify the "zebra" phenotype.

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