Characterization of a zebra Mutant of Rice with Increased Susceptibility to Light Stress

Kensuke Kusumi¹, Hisayo Komori¹, Hikaru Satoh² and Koh Iba¹, ³

¹ Department of Biology, Kyushu University, Hakozaki, Higashi-ku, Fukuoka, 812-8581 Japan
² Institute of Genetic Resources, Faculty of Agriculture, Kyushu-University, Hakozaki, Higashi-ku, Fukuoka, 812-8581 Japan

The rice zebra mutant TCM248 is a single recessive mutant. This mutant develops transverse-striped leaves with green and white sectors under alternate light/dark growth conditions. Mutants that were grown under a higher light intensity during the light period showed a more intense striped phenotype. The white tissues contained abnormal chloroplasts with few internal membrane structures, while the green tissues in the mutants contained normal chloroplasts. The white tissue contained only trace amounts of Chls and carotenoids, and mRNA accumulation of nuclear genes encoding chloroplast proteins (rbcS, cab) was strongly suppressed compared to that in the wild type plants. A series of growth condition shift experiments demonstrated that the mutant displayed the striped phenotype only if it was exposed to the alternate light/dark growth conditions during a limited stage of early leaf development. These data suggest that the zebra gene is involved in the acquisition of photoprotective capacity of the plants and that this gene functions at an early stage of chloroplast differentiation.

Key words: Chloroplast development — Leaf development — Oryza sativa — zebra mutant.

Photosynthetic organisms acclimate to changes in various environmental factors such as light, temperature, and nutrients by optimizing the photosynthetic apparatus. Light is a substrate for photosynthesis, but it is also a potential source of photooxidative damage to cellular and subcellular structures. When the absorbed light energy exceeds the rate of the photochemical reaction, photoinhibition, or inhibition of photosynthetic activity, occurs. Photoinhibition is a reversible process that does not directly cause chlorophyll bleaching or tissue damage. However, after a prolonged period of photoinhibition, the generated reactive oxygen species oxidatively damage plant cells, resulting in bleaching or photodestruction of chloroplast pigments and structural damage to chloroplasts and other organelles. Plants possess various protection systems against excess light stress, including regulation of pigment biosynthesis, the xanthophyll cycle, stabilization of light-harvesting system, and scavenging reactive oxygen species (Demming-Adams and Adams 1992, Foyer et al. 1994). These systems are very complex, and their efficiency is affected by the age of the plant (Kar et al. 1993, Corona et al. 1996, Falbel et al. 1996, Mock et al. 1998) as well as other environmental factors (Pastenes and Horton 1996, Thompson et al. 1989). However, the precise mechanism that regulates these processes is still unknown.

Many mutants with enhanced sensitivity to light stress have been identified (Niyogi 1999). These mutants develop nearly normal green leaves under low light conditions. However, under middle- or high-intensity light, they suffer necrotic or chlorotic damage. Destruction of chloroplast morphology, reduced PSI and PSII activity, and activation of antioxidant enzymes are generally observed in the damaged leaf cells (Niyogi 1999). These phenotypes in the mutants have been attributed to photooxidation due to destruction of the photoprotective machinery. In fact, some light-dependent necrotic/chlorotic mutants are deficient in a specific component of the photoprotective systems. For example, barley tigrina mutants have a defect in the regulation of chlorophyll biosynthesis and accumulate abnormally high amounts of protochlorophyllide in the dark (Nielsen 1974, Casadoro et al. 1983, Hansson et al. 1997). Under high-intensity light, the excess protochlorophyllide is photooxidized, leading to oxidative damage to the plant. The immutans mutant of Arabidopsis shows a light-dependent “variegated” phenotype. This mutant gene was cloned and found to encode a protein that seems to function in the electron transfer chain in chloroplasts (Carol et al. 1999, Wu et al. 1999). Analysis of these mutants provided a powerful tool for elucidating the mechanisms of the regulation of the photoprotection system in higher plants.

In this report we describe a rice mutant called zebra that is characterized by transverse green and chlorotic bands in the leaves. We found that the zebra mutation affects chloroplast differentiation only at a limited stage during early leaf development, resulting in light-dependent damage to leaf tissues. Within the chlorotic tissues of the mutant, carotenoid accumulation and the expression of some nuclear genes involved in the chloroplast biogenesis were suppressed. We propose that the zebra gene product contributes to the acquisition of the photoprotective ca-
capacity of the leaves during the early stages of chloroplast development.

**Materials and Methods**

**Plant material and growth conditions**—The *zebra* mutant line of rice (*Oryza sativa*), TCM248, was obtained by the mutation treatment of fertilized egg cells of a japonica rice cultivar Tai-chung 65 with N-methyl-N-nitrosourea (Satoh and Omura 1979). The japonica rice cultivar Kinmaze was used as the wild type reference strain. Seedlings were routinely grown in a growth chamber (Tokyo Rikakikai, Tokyo, Japan) under an artificial periodic condition of 12-h light (400 μmol m⁻² s⁻¹), 30°C/12-h dark, 20°C cycles, except for the experiments presented in Figures 1 and 4. A modified version of White's medium (Omura et al. 1977) containing 6% agar was used as the culture medium.

**Quantitative analysis of photosynthetic pigments**—Plant tissues were homogenized with a pestle and mortar, and the pigments were extracted from the homogenate twice with 80% acetone under dim light at room temperature. The Chl and total carotenoid contents were determined using the equation of Lichtenthaler (1987).

**Microscopic analysis**—Samples for electron microscopic examination were obtained from fully emerged third leaves of the wild-type and *zebra* seedlings. The leaf tissues were fixed, stained, and examined as described previously (Iba et al. 1991).

**RNA isolation and Northern blot analysis**—Total RNA was isolated according to the method of Ausubel et al. (1987). Each RNA sample was fractionated on a formaldehyde-agarose gel and blotted onto a nylon membrane (Pall Biosupport, NY). These membranes were then probed below as previously described (Kusumi et al. 1997) with the 32P-labeled DNA probes noted. RNA abundance was quantitated using a Fuji BAS-1000II Imaging System. The hybridization probes used in this study were: *cab*, pOSSLHC 2120, which contains the cDNA encoding the light-harvesting Chl protein of rice (Matsuoka 1990); *rbcS*, pOSSS 2106, which contains the cDNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase of rice (Matsuoka et al. 1988); *rbcL*, the 0.5-kb *Pvu*I fragment of the rice chloroplast gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase (Hirai et al. 1985); and *psbA*, the 3.9-kb * BamHI-BglII fragment from the rice chloroplast gene encoding the PSII protein (Hirai et al. 1985). A fragment of the rice cytosolic tRNA 56 gene (Reddy and Padayatty 1988) was amplified by the PCR with the primer pair 5'-GGC-ATC-TAA-GGG-TAG-TGG-GT-3' and 5'-CAT-AAA-TGC-ATC-AAG-TTG-CC-3'.

**Results**

The rice *zebra* mutant TCM248 developed leaves with chlorotic sectors under field conditions. The chlorotic sectors were not distributed randomly over the leaves; they were distributed as transverse stripes. The specific pattern of striping depended on the leaf size; the more-developed, larger leaves contained a large number of chlorotic stripes. Other characteristics of the mutant plants, such as the growth rate and the overall developmental patterns of the leaf, stem, and flower tissues, did not differ from those of the wild-type plants. In the mutant plants that were grown in growth chambers under the standard artificial periodical conditions of 12-h light (400 μmol m⁻² s⁻¹), 30°C/12-h dark, 20°C cycles, leaves with transverse chlorotic bands also developed (Fig. 1A). In the mutant plants that were grown either under a constant temperature condition (30°C) with 12-h light/12-h dark cycles or under continuous light with 12-h 30°C/12-h 20°C cycles, the same mutant phenotype was observed (Fig. 1B, C). However, under continuous illumination at 30°C, the mutant seedlings developed full-green leaves, and no chlorotic sectors were observed (Fig. 1D). These observations suggest that the periodic change in light and/or temperature is an essential environmental factor for manifestation of the *zebra* phenotype; therefore TCM248 is a light- and/or temperature-conditional mutant.

The chlorotic phenotype in TCM248 suggested that the levels of major pigments are reduced in the chlorotic tissues. Therefore, we measured the Chl and carotenoid contents in the wild-type and mutant leaves. In the TCM248 seedlings grown under the artificial periodic condition of 12-h light (400 μmol m⁻² s⁻¹), 30°C/12-h dark, 20°C cycles, the contents of Chl and carotenoids in the chlorotic tissues were each approximately 50% of the respective level in the green tissues of the mutant plants and were each approximately 30% of their respective levels in the wild-type plants (data not shown). The Chl a/b ratio in the chlorotic tissues of the mutant leaves and that in the wild-type leaves did not significantly differ (data not shown).

Electron microscopic observation showed that the leaf cells of TCM248 were heteroplastidic; they possessed both normal and abnormal chloroplasts (Fig. 2). In the cells of the green tissues of the mutant leaves, most of the chloroplasts were phenotypically normal with a typical lens shape and granal stacking (Fig. 2A, C). In contrast, a large proportion of the chloroplasts observed in chlorotic tissue had abnormal shapes (Fig. 2B). These abnormal chloroplasts possessed disorganized thylakoid membrane systems and contained small osmiophilic plastoglobules and many crystalloids (Fig. 2D).

Environmental factors, including light and temperature, affect plastid division (Hashimoto and Possingham 1989). However, the number of plastids in the wild-type leaf cells and that in the less-pigmented leaf cells of the mutant in our study did not differ significantly (data not shown). It is likely that the reduced pigment content in the chlorotic tissues of the *zebra* leaves is not due to blockage of plastid division or diminished number of chloroplasts.

The degree of chlorosis in the mutant leaves was enhanced by increasing the light intensity during plant development. In the mutant leaves that developed under artificial light/dark conditions with high-intensity light (2,000 μmol m⁻² s⁻¹) during the light period, the chlorotic sectors became more extensive, although the number of stripes in each leaf remained almost constant (Fig. 1E). In
Fig. 1 Phenotypes of the third (left) and fourth (right) leaves of *zebra* (A–F) and wild-type (G) seedlings grown for 15 d under the following conditions. (A and G) Standard periodic condition of 12-h light (400 μmol m$^{-2}$ s$^{-1}$), 30°C/12-h dark, 20°C cycles. (B) 12-h light/12-h dark cycles at a constant temperature (30°C). (C) Continuous light (400 μmol m$^{-2}$ s$^{-1}$) with 12-h 30°C/12-h 20°C cycles. (D) Continuous light (400 μmol m$^{-2}$ s$^{-1}$) at a constant temperature (30°C). (E) Standard periodic condition except that the light intensity was 2,000 μmol m$^{-2}$ s$^{-1}$. (F) Standard periodic condition except that the light intensity was 2.0 μmol m$^{-2}$ s$^{-1}$.

In contrast, when the light intensity during the light period was reduced to 2.0 μmol m$^{-2}$ s$^{-1}$, TCM248 developed full-green leaves, and the stripes were not observed (Fig. 1F). The fact that the *zebra* phenotype is expressed in the TCM248 mutant under middle- and high-intensity light but not under low-intensity light implies that the underlying mechanism of the *zebra* phenotype is photooxygenation due to a disturbance in the photoprotective machinery. The reduced accumulation of carotenoids in the mutant leaves, which play a fundamental role in dissipating excess light
Fig. 2 Fine structure of mesophyll cells (A, B) and plastids (C, D) in the fully-expanded third leaves of the zebra mutant. (A, C) Mesophyll cells and representative plastids in the green tissues. (B, D) Mesophyll cells and representative plastids in the chlorotic tissues. Seedlings were grown under the standard periodic condition described in Fig. 1A. Bar = 1 μm.

energy (Demming-Adams and Adams 1992), is expected to preferentially cause photooxidative damage under high-intensity light. However, when grown under a continuous dim-light condition (2.0 μmol m$^{-2}$ s$^{-1}$) under which photooxidative damage does not occur, the levels of Chl and carotenoids in the TCM248 mutants were similar to those in the wild-type plants grown under the same condition (data not shown). This is in contrast with known carotenoid-deficient plants (Mayfield and Taylor 1984, Susek and Chory 1992, Wetzel et al. 1994). It seems that the reduced carotenoid content in the chlorotic tissues of TCM248 grown under normal- or high-intensity light is due to a secondary effect of photooxidation, not to specific blockage in the carotenoid biosynthetic pathway.

To analyze how the zebra mutation affects the expression of nuclear and plastid genes encoding chloroplast components, the steady-state mRNA levels of several genes in the wild-type and mutant plants grown under the standard periodic light/dark cycles were compared. Total RNA was isolated from the wild-type and TCM248 chlorotic leaf tissues. To eliminate contamination by phenotypically-normal (green) leaf tissues, we dissected the visually pure-white tissues from the mutant leaves and used these as the mutant samples. The genes that were examined included two nuclear-encoded genes, $cab$ and $rbcS$, and two plastid-encoded genes, $psbA$ and $rbcL$. As shown in Figure 3, the chlorotic tissues of TCM248 contained high amounts of $psbA$ and $rbcL$ transcripts. In contrast, $cab$ and $rbcS$ transcripts were barely detectable in the chlorotic tissues. Quantitative analysis revealed that the mRNA levels of the two plastid genes in the chlorotic tissues were approximately 70% of their respective levels in the wild-type plants, whereas the mRNA levels of the two nuclear genes did not exceed 10% of their respective levels in the wild type.

We have preliminarily confirmed that expression of the zebra phenotype in the mutant leaf is not influenced by growth conditions after its expansion. This suggests that the leaf phenotype is irreversibly determined at a certain stage of leaf development before its maturation. In order to delimit the developmental stage during which the phenotype is determined, a series of experiments of shifting the growth conditions was conducted in the third leaves (Fig. 4). In the shift experiment that consisted of a change in the condition from constant temperature and light to the standard periodic condition, the mutant seedlings developed third leaves with chlorotic bands when the seedlings were shifted at the stage of leaf age 2.0 or earlier (Fig. 4A). In contrast, in the shift experiment that consisted of a change in the condition from the standard periodic condition to a constant condition of temperature and light, seedlings that were shifted at a leaf age of 3.0 or later deve-
Fig. 3 Steady-state RNA level of nuclear and plastid genes in leaves of the wild type and zebra mutant. Seedlings were grown under the standard periodic condition noted in Figure 1A. RNA was isolated from mature, fully expanded leaves. Each lane of the gel contains 1.25 or 12.5 μg of total RNA as indicated above. The blot was hybridized to probes of the nuclear genes, cab and rbcS, and the plastid genes, rbcL and psbA. A single filter was probed sequentially with each probe. The corresponding ethidium bromide-stained gel is shown at the bottom, and the position of the rRNAs (18S and 25S) is indicated to the left of the gel.

The wild-type control seedlings developed full green leaves under all treatments. Taken together, these results suggest that the phenotype of the third leaf is determined during the period between the emergence of the third and fourth leaves in TCM248.

Discussion

The expression of the zebra phenotype is influenced by the light intensity. Although light is the energy source for photosynthesis, it also can be harmful to plants. The absorbance of excess light energy by photosynthetic pigments causes photodestruction, resulting in the concomitant destruction of chloroplasts and reduction in Chl concentration. In addition, such damage blocks the expression of nuclear genes such as cab and rbcS that are necessary for photosynthesis (Mayfield and Taylor 1984, Oelmüller 1989, Susek and Chory 1992). For example, the herbicide Norflurazon blocks carotenoid biosynthesis, thereby inducing the photooxidation of chloroplasts and suppression of cab and rbcS expression. These characteristics were seen in TCM248 grown under normal- or high-light intensity but not in those grown under low-light intensity, suggesting that the zebra mutation is phenotypically expressed through photooxidation caused by excess light energy.

A striking characteristic of TCM248 was the trans-
verse striping of the leaves that was expressed under periodic light-dark cycles. In monocotyledonous plants, the leaf that emerges from the leaf sheath has a linear gradient of cellular and chloroplast development between the leaf base and the leaf tip (Mullet 1993, Kusumi et al. 1997). Therefore, the chlorotic stripe in TCM248 is thought to be formed in response to the occurrence of a periodic change in some environmental factor (temperature and/or light) during a specific stage of leaf cell development. In this context, the phenotype of TCM248 is similar to that of the *tigrina* mutants of barley, which are also characterized by alternate transverse green and necrotic bands in the leaf (Nielsen 1974). However, the *tigrina* mutants accumulate 2 to 10 times the wild-type amount of tigrina proteins in the leaf (Nielsen 1974). While TCM248 accumulated the same amount of protochlorophyllide during the dark period (Nielsen 1974), while TCM248 accumulated the same amount of protochlorophyllide as the wild type (data not shown).

Almost all the chloroplasts that were observed in the green areas of TCM248 leaves had well-developed thylakoid membrane systems similar to those seen in wild-type leaves, while the cells in the chlorotic tissues of the mutant had many abnormal plastids (Fig. 2). Since the other morphological characteristics of TCM248 were nearly normal, the zebra gene product most likely acts at a particular step of chloroplast differentiation. Based on studies of monocotyledonous plants such as rice and barley, the process of chloroplast differentiation can be divided into several steps (Chory 1991, Mullet 1993, Kusumi et al. 1997). The first step involves the activation of plastid DNA synthesis. The number and size of chloroplasts are determined by the end of this step. The second step is characterized by an increase in plastid transcription activity; the expression of plastid transcription/translation machinery occurs during this stage. During the final step, the photosynthetic apparatus, which is encoded by both nuclear and plastid genes, is synthesized at high levels and assembled to form active and functional photosynthetic machinery. The chloroplasts observed in TCM248 were defective, but they seemed to be partially developed; they were nearly normal in size and contained rudimentary thylakoids (Fig. 2). In addition, there was no significant difference in the number of chloroplasts per cell between the mutant and wild-type leaf cells (data not shown). Furthermore, the genetic systems of the chloroplast in the mutant did not significantly differ from that in the wild type (Fig. 3). These observations indicate that the initial steps of chloroplast differentiation from proplastids occur in TCM248 leaves, but, at a later step, photooxidative damage arrests chloroplast differentiation.

Anatomical studies of rice have shown that the shoot always contains four immature leaves (including a leaf primordium) at different developmental stages when a leaf begins to emerge from the adjacent lower leaf sheath, irrespective of the environmental condition or the growth stage of the plant (Yamazaki 1963, Nemoto and Yamazaki 1993). These immature and emerging leaves go through a series of successive stages of leaf development, starting with P1 (leaf primordium) through P2, P3, P4, P5 and P6 (fully expanded leaf). When the second leaf begins to emerge, it corresponds to P5, and the third leaf just adjacent to it corresponds to P4. Our shift experiments showed that the zebra mutation affects the pigmentation of the third leaves of TCM248 during the time period between the emergence of the third and fourth leaves (Fig. 4). In the classification described by Yamazaki (1963), this period corresponds to P5. At this stage, primary leaf cell division and the formation of basic leaf structure are completed, and the leaf starts to emerge mainly by cell elongation and the activity of the intercalary meristem. While the developmental state of the chloroplast in leaves at the P5 stage is not yet known, our previous study has shown that the expression of genes encoding the photosynthetic apparatus starts at the P5 stage (Kusumi et al. 1997). In addition, wild-type developing leaves corresponding to P5 were visually green, whereas those corresponding to early P4 were pure white (data not shown), suggesting that the formation of the thylakoid membrane and the subsequent greening process occurs during the P5 stage. These observations suggest that the zebra gene functions in the formation of protection machinery against photooxidative damage; this argument is further supported by the fact that these mechanisms seem to be closely associated with the formation of the thylakoid membrane. In fact, it has been reported in tobacco that the transcripts of antioxidative enzymes such as catalases, superoxide dismutases, and glutathione peroxidase are most abundant in young emerging leaves (Mock et al. 1998). The activation of genes involved in carotenoid biosynthesis in the chloroplast has also been studied during this stage (Corona et al. 1996).

Although damage to the photosynthetic apparatus in chloroplasts is widely considered to be the primary response of leaves to light stress, details of the protection system and the mechanisms that regulate the photoprotective responses are still poorly understood. Application of normal- or high-intensity light to a zebra mutant on a light-dark cycle induced photooxidative damage to the leaf only when it was applied during a limited developmental stage. This mutant is an attractive system for elucidating the formation of the photoprotection capacity in plants and the nuclear-organelle interactions during tissue and organ development.

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