Plant Physiol. (1996) 110: 997–1005

Cool-Temperature-Induced Chlorosis in Rice Plants

I. Relationship between the Induction and a Disturbance of Etioplast Development

Riichiro Yoshida*, Akira Kanno, Tadashi Sato, and Toshiaki Kameya

Institute of Genetic Ecology, Tohoku University, Aoba-ku, Sendai 980–77, Japan

We have established an experimental system for mimicking the phenomenon of cool-temperature-induced chlorosis (CTIC) in rice plants (*Oryza sativa* L.). Rice seedlings were initially grown in darkness under cool-temperature conditions and then exposed to light and warm conditions to follow the expression of CTIC. Induction of CTIC in the sensitive cultivar (cv Surjamukhi) was bimodally dependent on the temperatures experienced during the initial growth in darkness. CTIC was maximally induced between 15 and 17°C. A positive correlation was demonstrated between induction of CTIC and the growth activity of shoots during growth in darkness. Electrophoretic and immunoblot analysis revealed that accumulation of NADPH-protochlorophyllide oxidoreductase in plastids was also bimodally dependent on the temperatures during the growth in darkness with minimum accumulation between 15 and 17°C, suggesting that the reduction of NADPH-protochlorophyllide oxidoreductase accumulation in plastids might be closely linked to a disturbance in transformations of plastids to etioplasts during the dark growth under the critical temperatures and thereby to the CTIC phenomenon. This was corroborated by electron microscopic observations. These results suggest that growth is one of the determining factors for the expression of CTIC phenotype in rice under cool temperature.

Plants that are indigenous to tropical and subtropical climates are generally sensitive to low, above-freezing temperatures. Rice (*Oryza sativa* L.) is a typical plant of tropical origin and is susceptible to temperatures below 20°C. Various types of chilling injury have been surveyed and reported in rice plants. CTIC in developing young leaves is one symptom of chilling injury (Choung and Omura, 1982; Kaimori and Takahashi, 1985). When seedlings of rice are exposed to cool weather, the newly emerging leaves lack Chl. Once such chlorotic leaves have developed, they remain white, even under permissive temperature conditions, without withering. Thus, the expression of CTIC is distinct from the irreversible injury to cells caused by low temperatures. Cultivars sensitive and resistant to conditions that induce CTIC have been identified among numerous Asian rice cultivars. Almost all cultivars of the *Japonica* type are resistant, whereas *Indica* cultivars are extremely sensitive (Choung and Omura, 1982). The inheritance of susceptibility to CTIC is considered to be governed by simple genetic factors, and a few major genes are involved in the CTIC-susceptible phenotype (Choung and Omura, 1982).

CTIC can be induced by cooling the basal part of a leaf sheath in which immature young leaves and meristems are developing (Kaimori and Takahashi, 1985). Since in monocots leaf cells develop primarily from meristem tissue located in the basal part of the leaves (Boffey et al., 1979), immature leaves that are already present within the leaf sheath are considered to be the site of greatest sensitivity to cool temperatures and CTIC.

Many cold-sensitive, Chl-deficient mutants of monocot plants have been isolated and studied. In studies with the M11 mutant of maize (*Zea mays* L.), the primary site for cold sensitivity is considered to be the shoot apex, where new leaves are developing and the rapid expansion of cells is occurring (Millerd and McWilliam, 1968). The cited authors concluded that the absence of Chl in developing leaves was due to the impaired development of functional chloroplasts at a low temperature and an increased sensitivity to photooxidation. The virescent mutant *v16/v16* of maize also exhibits extreme chlorosis when grown at temperatures below 25°C but without any photooxidation (Hopkins, 1982; Hopkins and Elfman, 1984). Iba et al. (1991) and Kusumi et al. (1994) reported that, in a virescent mutant of rice, the transformation of undifferentiated plastids to functional chloroplasts and/or the Chl-biosynthetic pathway was inhibited by the action of cool temperatures on the so-called virescent gene. This gene is expressed immediately after the growth stage at which leaf cells differentiate and generate the basic structure of the leaf.

During the differentiation and growth of leaf cells, proplastids differentiate into chloroplasts in synchrony with cell growth (see refs. cited by Mullet, 1988). Based on the results as reported, it is hypothesized that the CTIC phenomenon in rice plants might be closely related to the developmental processes of plastids and the coordinated expression of genes, being controlled by two factors, namely the active growth of the immature leaves within the base of the leaf sheath and the cool temperature per se.

In an attempt to characterize the mechanism of the expression of CTIC in rice plants, we examined the changes in profiles of plastid proteins, in particular, nuclear-encoded protein of 36-kD POR, as a possible marker for the trans-

* Corresponding author; e-mail riichi@bansui.ige.tohoku.ac.jp; fax 81-22-263-9845.

Abbreviations: CTIC, cool-temperature-induced chlorosis; O-195, Ouu No. 195; POR, NADPH-Pchlide oxidoreductase; SUR, Surjamukhi.

formation of undifferentiated plastids into etioplasts (Ryberg and Sundqvist, 1982; Dehesh and Ryberg, 1985), as well as ultrastructural changes in plastids in newly developed young leaves. For the study we used a newly established model system for mimicking CTIC in rice plants in the laboratory. Evidence is presented that CTIC expression in sensitive rice cultivars is closely associated with an interference in the development of plastids into normal etioplasts in accordance with leaf cell growth under a certain range of cool temperatures.

MATERIALS AND METHODS

Plant Materials

Rice cv SUR and cv O-195 were used as CTIC-sensitive and CTIC-resistant materials, respectively. SUR is a native cultivar from the Bengal area in India, and O-195 is a *Japonica* cultivar.

Growth Conditions

Seeds of each cultivar were submerged in water at 25°C for 24 h and then they were sterilized with 70% ethanol for 30 s and with 1% $HClO_3$ for 15 min. The sterilized seeds were germinated on wet filter paper in Petri dishes in darkness at 25°C. Germinated seeds with plumules of about 1 mm in length were selected and used for experiments. At this stage, second-leaf primodia already exist inside the premules and are beginning to elongate (Hoshikawa, 1989). Two germinated seeds were transplanted to agar medium consisting of modified Hoagland solution [0.75 mM KNO_3, 0.5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 mM $NH_4H_2PO_4$, 0.25 mM $MgSO_4 \cdot 7H_2O$, 6.25 μM KCl, 3.125 μM H_3BO_3, 0.25 μM $MuSO_4 \cdot H_2O$, 0.25 μM $ZnSO_4 \cdot 7H_2O$, 62.5 nM $CuSO_4 \cdot 5H_2O$, 62.5 nM H_2MoO_4, and 2.5 μM Fe-EDTA] and 0.4% agar. After 10 d of dark growth at 10, 13, 15, or 17°C, seedlings were transferred to darkness at 25°C. They were grown under these conditions until the base of second leaf blades had emerged completely from the coleoptiles. To bring seedlings to this growth stage, dark growth at 20 and 25°C was continued until the seedlings had reached the same stage as mentioned above, i.e. about 8 d at 20°C and about 4 d at 25°C. The seedlings, all of the same growth stage, were then illuminated for 24 h at 25°C to allow accumulation of Chl and expression of CTIC. Three fluorescent tubes (FL10·BR, 20 W; Matsushita Electronic Co., Tokyo, Japan) were used as the light source (light intensity, 28.4 μmol m^{-2} s^{-1}).

Isolation of Plastids

The second leaf blades of dark-grown seedlings were cut into small pieces and ground with a motor and pestle in grinding medium that contained 330 mM mannitol, 40 mM Hepes-KOH (pH 8.0), and 2 mM EDTA. The homogenate was filtered through eight layers of gauze and centrifuged for 10 min at 500*g*. The supernatant was centrifuged for 10 min at 2500*g*, and then the plastid-enriched pellet was resuspended in the grinding medium and centrifuged again for 10 min at 2500*g*. All manipulations were performed at 0°C. The final pellet was designated the plastid-enriched fraction. The extent of enrichment was examined under a phase-contrast microscope.

SDS-PAGE

SDS-PAGE on a 12% (w/v) polyacrylamide gel was performed by the method of Laemmli (1970). An equal volume of SDS-lysis buffer (100 mM Tris-HCl, pH 6.8, 100 mM DTT, 4% SDS, 0.2% bromphenol blue, 20% glycerol) was added to an aliquot of the samples of plastids, and proteins were solubilized by heating at 75°C for 10 min. The solubilized sample was centrifuged at 10,000*g* for 15 min prior to electrophoresis. Electrophoresis was performed at a constant current of 15 mA. Protein bands were visualized by a silver-staining method (Wray et al., 1981). Proteins were quantified by the method of Bradford (1976) with BSA as the standard.

Western Blots

For immunoblotting, proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham) with a semidry blotting apparatus (Horizblot; ATTO, Tokyo, Japan) by the standard procedure (Harlow and Lane, 1988). The membrane sheet was blocked with 10% skim milk prior to reaction with primary antibodies and then rinsed with three changes of PBS (pH 7.5) that contained 0.1% Tween 20. Binding of antibodies was detected by a nonradioactive chemiluminescence method (ECL Western blotting detection kit, Amersham) using horseradish peroxidase-coupled second antibodies. Antiserum against POR was raised against an amino acid sequence that is conserved in PORs from *Hordeum vulgare* (Schultz et al., 1989), *Pisum sativum* (Spano et al., 1992), and *Pinus mugo* (Forreiter and Apel, 1993), namely TMQEFHRRYHEETGI (Fig. 1), which was synthesized with a multiple antigen peptide system (Tam, 1988) (Sawady Technology, Tokyo, Japan). Levels of antigens on immunoblots were quantified by densitometric scanning with an automated analyzer (model EPA-3000; Maruzen Petrochemical, Tokyo, Japan).

EM for Analysis of Plastid Development

The middle part of the second leaf blade of an etiolated seedling was cut into small pieces (1 × 1 mm^2) and fixed in 2.5% (w/v) glutaraldehyde in 50 mM Tris-HCl solution (pH 7.0) overnight at 4°C. After fixation with osmic acid for 6 h at 4°C, samples were dehydrated in a graded ethanol series, substituted with propylene oxide, and embedded in Epon 812 (TAAB, Berkshire, UK). Thin sections (0.1 μm)

H. vulgare	---	SKVCNMLTMQEFHRRYEETGITFASLYPG	295
P. sativum	---	SKVCNMLTMQEFHRRYEETGITFASLYPG	306
P. mugo	---	SKVCNMLTMQEFHRRYEETGITFASLYPG	307

Figure 1. The consensus amino acid sequence of POR that is conserved in different plant species. The conserved shaded sequence was used to raise antiserum against POR. Experimental details are given in "Materials and Methods."

were stained with uranyl acetate and lead citrate and then observed with an electron microscope (JEM1200EX, JEOL).

Extraction of Chl

For the efficient extraction of Chl from small samples, we referred to the report of Moran and Porath (1980) and used dimethylformaldehyde as the extraction solvent. Upper parts of seedlings, except for the mesocotyls and the coleoptiles, were cut into small pieces and placed into test tubes, and then 10 mL of dimethylformaldehyde were added. After a 24-h incubation at 4°C in darkness, the A_{647} and A_{664} of each extract were measured. Chl content was quantified by application of the equation of Moran (1982).

RESULTS

Greening of Dark-Grown Seedlings upon Illumination under Warm Conditions

In the CTIC-sensitive cv SUR, the greening process upon illumination at 25°C was distinctly inhibited by initial growth at temperatures below 20°C and above 13°C (Fig. 2). Chl content of such seedlings was about 20% of those in control seedlings, which had previously been grown at 25°C in darkness. It was interesting that inhibition of the greening process was partially mitigated as the temperature for initial growth step decreased below 15°C (Fig. 2). Thus, the profile of inhibition of subsequent greening as a function of temperature during prior growth in darkness was bimodal: below and above the most critical range of temperatures, namely between 15 and 17°C, inhibition was less extensive. This feature might be important with respect to the characterization of the molecular mechanism of the expression of CTIC in rice plants.

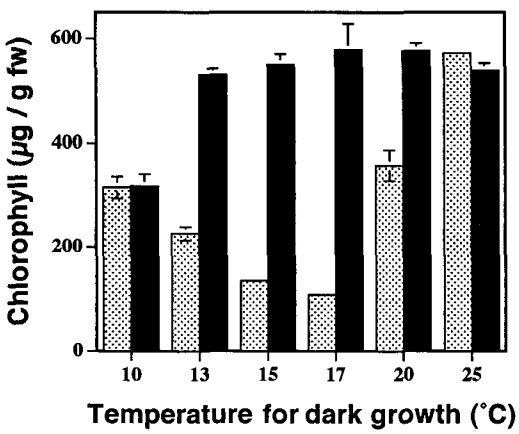

Figure 2. Effects of temperature during initial growth in darkness on the accumulation of Chl during subsequent illumination. Seedlings were grown in the dark at 10, 13, 15, and 17°C for 10 d and then transferred to 25°C in darkness until the second leaf had completely emerged from the leaf sheath. Dark growth at 20 or 25°C was continued for 8 or 4 d, respectively, until the second leaves had completely emerged. These seedlings, in all at the same stage of growth, were illuminated for 24 h at 25°C. ■, O-195; ▨, SUR. Each bar shows the mean ± SE (n = 4). The missing error bars indicate that they are smaller than the label marks. fw, Fresh weight.

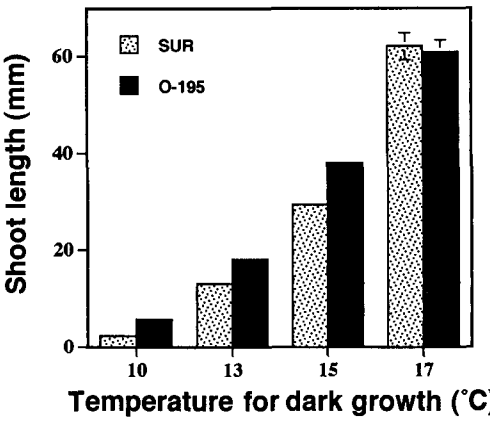

Figure 3. Growth of shoots after 10 d of growth in darkness at cool temperatures. Each bar shows the mean ± SE (n = 4). The missing error bars indicate that they are smaller than the label marks.

In the CTIC-resistant cv O-195, by contrast, no inhibition was observed of either greening or accumulation of Chl upon illumination unless the temperature for the initial growth step had been below 13°C. When the initial growth step was at 10°C, subsequent greening was significantly inhibited, and the extent of inhibition was similar to that in the seedlings of the CTIC-sensitive cv SUR (Fig. 2).

Relationship between Shoot Growth and Induction of CTIC

We examined the relationship between the expression of CTIC and the extent of shoot growth in the initial growth step at different temperatures for 10 d. A difference was clearly observed with respect to the growth of shoots among seedlings that had been grown at different temperatures in the initial growth step (Fig. 3). In SUR, shoots increased in length by about 30 mm during the initial growth in darkness at 15°C. However, shoot growth was markedly suppressed at 13 and 10°C, with increases of only 13 and 2 mm, respectively (Fig. 3). Thus, the bimodal induction of CTIC seems to be closely related to the extent of shoot growth under cool conditions.

The time courses of the shoot growth and the induction of CTIC were examined during growth at 15°C in darkness. Under the cool condition, shoots grew slowly, at a rate of 1.5 mm/d for 4 d, and then the rate increased to 4.7 mm/d and shoots grew steadily at this rate until d 10 (Fig. 4A). The accumulation of Chl proceeded normally for 4 d of growth without a detectable sign of CTIC (Fig. 4B). After 5 d of growth, however, white spots began to appear on the developed leaves and Chl content declined abruptly as a function of growth period. The results suggest that a successive growth of shoots for more than 5 d under the cool condition is required for the induction of CTIC in rice.

Electrophoretic Patterns of Plastid Proteins and Specificity of the Antiserum Raised against a Fragment of POR

Observations under the phase-contrast microscope revealed that the pellets obtained after differential centrifu-

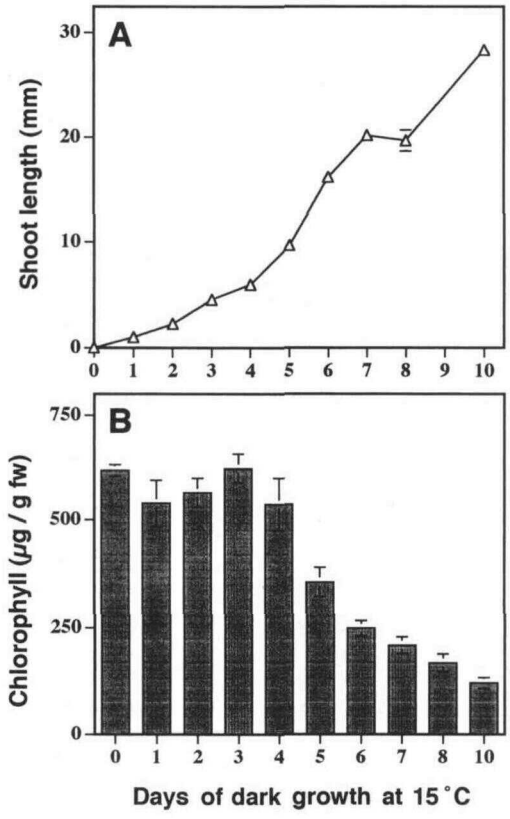

Figure 4. Relationship between growth of shoots of SUR seedlings during the course of dark growth at 15°C and the accumulation of Chl upon subsequent illumination at 25°C. Shoot length was measured from the top to the base. A, Growth of shoots; B, accumulation of Chl. Each bar and point represents the mean ± SE (n = 4). The missing error bars in A indicate that they are smaller than the label marks. fw, Fresh weight.

gation were relatively enriched with respect to plastids, either undifferentiated plastids or etioplasts, with limited contamination by nuclei and cell debris (data not shown). Electrophoresis of the proteins solubilized from the plastid-enriched fractions prepared from the second leaves of rice seedlings that had been grown at 25°C in darkness revealed that plastids of the two cultivars had a common polypeptide composition (Fig. 5A). One of the major polypeptides was expected to correspond to POR, since this enzyme is known to be abundant in the prolamellar bodies of etioplasts (Ryberg and Sundqvist, 1982; Dehesh and Ryberg, 1985). Antiserum raised against an amino acid sequence that is conserved in PORs in different species cross-reacted specifically with a major polypeptide of 36 kD in the etioplast fractions isolated from dark-grown rice seedlings (Fig. 5B).

Changes in the Level of the 36-kD Polypeptide upon Illumination

Given that the 36-kD polypeptide corresponded to POR, we expected that the level of the polypeptide would be reduced markedly upon illumination of dark-grown seedlings (Apel, 1981; Santel and Apel, 1981; Dehesh et al., 1986a, 1986b; Häuser et al., 1987; Schultz and Senger, 1993). Therefore, 5-d-old etiolated seedlings of SUR, which had been grown at 25°C in darkness, were transferred to light and warm conditions, and the level of the 36-kD polypeptide was monitored. Plastid-enriched fractions were prepared from the second leaves after illumination for given periods of time and were subjected to SDS-PAGE and immunoblot analysis (Fig. 6). It was clear that the intensity of staining of the band of the 36-kD polypeptide declined abruptly even after illumination for as little as 2 h, as assessed by both silver staining of the gel and immunoblotting. After prolonged illumination for up to 24 h, the polypeptide became barely detectable by either silver staining or immunoblotting. These results suggest that the 36-kD polypeptide in the plastid-enriched fractions from

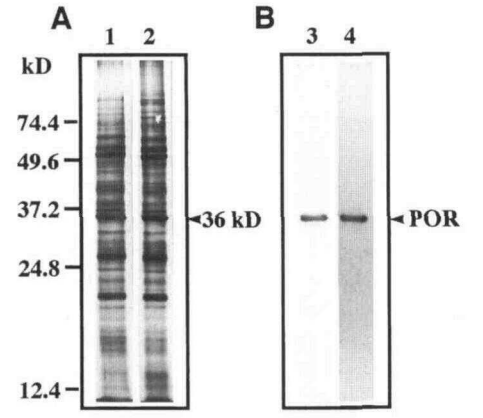

Figure 5. Specificity of the antiserum against POR. A plastid-enriched fraction was prepared from etiolated seedlings that had been grown for 5 d at 25°C in the darkness. Total proteins, solubilized from the plastid-enriched fraction, were analyzed by SDS-PAGE (A) and immunoblotting (B). Lanes 1 and 3, O-195; lanes 2 and 4, SUR. Three micrograms of protein were fractionated in each lane.

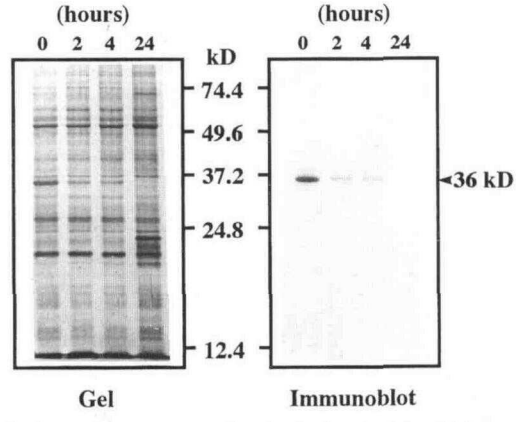

Figure 6. Light-dependent decline in the level of the 36-kD polypeptide in plastid-enriched fractions. Seedlings of SUR were grown at 25°C for 5 d in darkness and then exposed to illumination at 25°C. Plastid-enriched fractions were prepared from the second leaves immediately before (zero time) and after illumination for given times. Total proteins were solubilized and analyzed by SDS-PAGE and immunoblotting. Three micrograms of protein were fractionated in each lane.

etiolated rice seedlings that had been grown at a normal temperature in darkness was identical with the etioplast-abundant protein, POR.

Changes in the Level of the 36-kD POR Related to the Growth Temperature in Darkness

Total proteins were solubilized from the plastid preparations and were subjected to SDS-PAGE. With the exception of changes in level of the 36-kD polypeptide, no detectable change was observed in either cultivar in the electrophoretic pattern of the total plastid proteins with changes in growth temperatures (Fig. 7). A major difference was detected in the changes in intensity of staining of the 36-kD polypeptide as a function of growth temperature between seedlings of cultivars that were resistant and sensitive to CTIC. In plastid-enriched fractions from SUR seedlings, the intensity of staining of the 36-kD polypeptide decreased markedly as the temperature for initial growth step was lowered to the most effective range for induction of CTIC, namely 15 to 17°C (Figs. 2 and 7). We also noted that the intensity of staining of the 36-kD protein increased with further decreases in the temperature for the initial growth step below 15°C. By contrast, in the CTIC-resistant cultivar O-195, no detectable changes were observed in the intensity of staining of the band of the 36-kD polypeptide irrespective of the temperature for initial growth step. Immunoblot analysis confirmed that the growth temperature-dependent changes in the intensity of staining of the 36-kD polypeptide coincided closely with the changes in the levels of rice POR protein (Fig. 7). Densitometric scanning of the immunoblots indicated that the level of POR in SUR decreased with decreases in the growth temperature to 15°C, and then the level increased with further decreases in the temperature to 10°C (Fig. 8). The relative level of POR in SUR after growth at 15°C was about 25% of that in control seedlings that had been grown at 25°C, whereas the relative level was almost the same as that in the control when seedlings were grown at 10°C. In the CTIC-resistant cultivar O-195, by contrast, the level of POR was almost constant throughout the range of tested growth temperature (Figs. 7 and 8).

Ultrastructural Changes in Plastids as a Function of Growth Temperature

Since it had become evident from our analysis that the accumulation of the 36-kD POR protein in the CTIC-sensitive cultivar was substantially inhibited when the growth temperature was within the critical range, we postulated

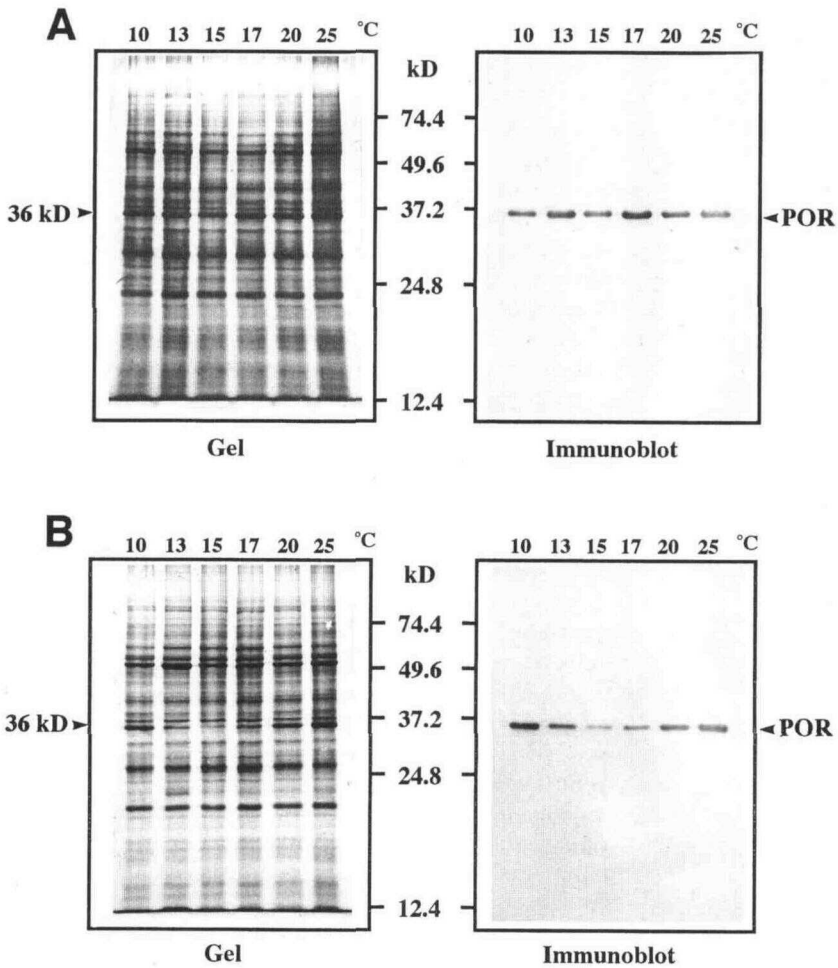

Figure 7. Changes in profiles after SDS-PAGE and immunoblotting of polypeptides in plastid-enriched fractions as a function of the growth temperature in darkness. Young seedlings with 1-mm plumules were first grown in the dark at different temperatures below 17°C and then transferred to dark and warm conditions and grown until they reached the second-leaf stage. In the case of temperatures of 20°C and above, seedlings were grown continuously in darkness for 8 d (20°C) or for 5 d (25°C) until they reached the same growth stage as above. Plastid-enriched fractions were prepared from the second leaves of these seedlings. Three micrograms of protein were fractionated in each lane. Gels were either stained with silver or blotted onto a polyvinylidene difluoride membrane and immunostained with the POR-specific antiserum. A, O-195; B, SUR.

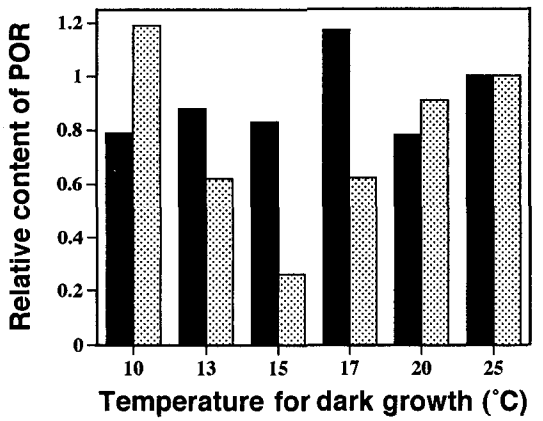

Figure 8. Densitometric analysis of levels of POR in plastid-enriched fractions during growth in darkness at different temperatures. The conditions for growth and protein analysis were the same as described in the legend to Figure 5. The intensity of bands of POR were determined by densitometric scanning of the immunoblots in Figure 5 and results are expressed relative to the results for the second leaves of seedlings that were grown at 25°C in darkness. ■, O-195; ▨, SUR.

that the development of plastids into normal etioplasts might be irreversibly interrupted by growth at the cool temperatures. Electron microscopic studies were performed to examine the development of plastids into etioplasts in the second leaves of seedlings that had been grown at cool (15°C) and normal (25°C) temperatures in the initial growth step. Figure 9, A and E, clearly shows the existence of prolamellar bodies and prothylakoid membranes, both of which are characteristic of normal etioplasts, in plastids from the second leaves of both cultivars when seedlings were grown at 25°C in darkness. Upon transfer of such seedlings to light and warm conditions for 24 h, prolamellar bodies disappeared and prothylakoid membranes developed further into normal thylakoids, in association with greening of leaves (Fig. 9, C and G). In the seedlings of the CTIC-resistant cultivar O-195, which had been grown at 15°C (in the initial growth step) and then transferred to light and warm conditions, prolamellar bodies disappeared and prothylakoid membranes developed into thylakoids. However, the extent of development of thylakoids was not as great as in the control seedlings grown at 25°C (Fig. 9D). By contrast, in the second leaves of the CTIC-sensitive seedlings, which had been grown in darkness at 15°C, we could not confirm any prolamellar bodies and prothylakoid membranes (Fig. 9F). No apparent changes were observed in the plastids upon transfer of such seedlings to light and warm conditions (Fig. 9H).

DISCUSSION

The phenomenon of CTIC is restricted to a small number of leaves that are newly developing from the base of the leaf sheath after a period of cool weather and, therefore, it is not fatal to the plant as a whole. However, CTIC can cause significant retardation of growth and a reduction in crop productivity. In spite of the importance of CTIC in crop production, very little is known about the physiological and molecular basis of this problem. To gain insight into the mechanism of CTIC, we established a model system for mimicking in the laboratory the phenomenon of CTIC that occurs in the field conditions. In the model system, the growth of immature leaves and the development of undifferentiated plastids to etioplasts in darkness were under the influence of cool temperatures. Upon subsequent transfer to light and warm conditions, the development of etioplasts to normal chloroplasts was expected to be influenced by the previous growth under cool and dark conditions. One of the most interesting findings in our study was that the expression of CTIC in the sensitive cv SUR was bimodally dependent on the temperature during the first growth in darkness. Since shoots grew at different rates in darkness, depending on the environmental temperature, the induction of CTIC in SUR might have been controlled by two factors, namely the active growth of shoots and/or immature leaves inside the base of the leaf sheath and the cool temperature.

In the case of the chilling-sensitive Arabidopsis mutant PM11, the plants are not visibly damaged at 5°C, but they rapidly become chlorotic at 13°C (Schneider et al., 1995). Although it is not clear whether this type of chlorosis occurs in newly developing leaves or in already developed leaves, this observation is in accordance with the bimodal response of the rice seedlings to growth temperature observed in this study. In general, the severity of chilling injury in sensitive plants increases as the temperature is lowered and with prolonged exposure (Lyons, 1973). However, the expression of CTIC in sensitive rice cultivars cannot be explained in terms of chilling injuries that lead to cell death. Genetic studies have also indicated that in rice plants the genes that control chilling injury, with withering of leaves, and the genes that are associated with expression of CTIC are located at different loci (Nagamine, 1991).

To elucidate the mechanism of the induction of CTIC in rice from a biochemical point of view, we investigated the effects of cool temperatures on the development of plastids, with special reference to the level of POR, which has been well documented to accumulate abundantly in etioplasts (Ryberg and Sundqvist, 1982; Dehesh and Ryberg, 1985). Immunoblot analysis of 36-kD POR protein in the CTIC-sensitive cultivar suggested that the development of plastids to etioplasts was specifically inhibited by a narrow range of cool temperatures and that the inhibition was also under the control of leaf cell growth. This suggestion was also corroborated by electron microscopic observations.

From the results obtained in the present study, it appeared that the induction of CTIC was closely related to the development of plastids in the immature second leaf inside the leaf sheath under the cool conditions, and such development is also under the control of cell growth (Kirk and Tilney-Basset, 1978; Mullet, 1988). In photosynthetic tissues or cells, proplastids develop into functional chloroplasts and the process requires the coordinated expression of plastid- and nuclear-encoded genes (Taylor, 1989; Susek et al., 1993), the biosynthesis of photosynthetic pigments (Beale and Weinstein, 1990; He et al., 1994), and structural

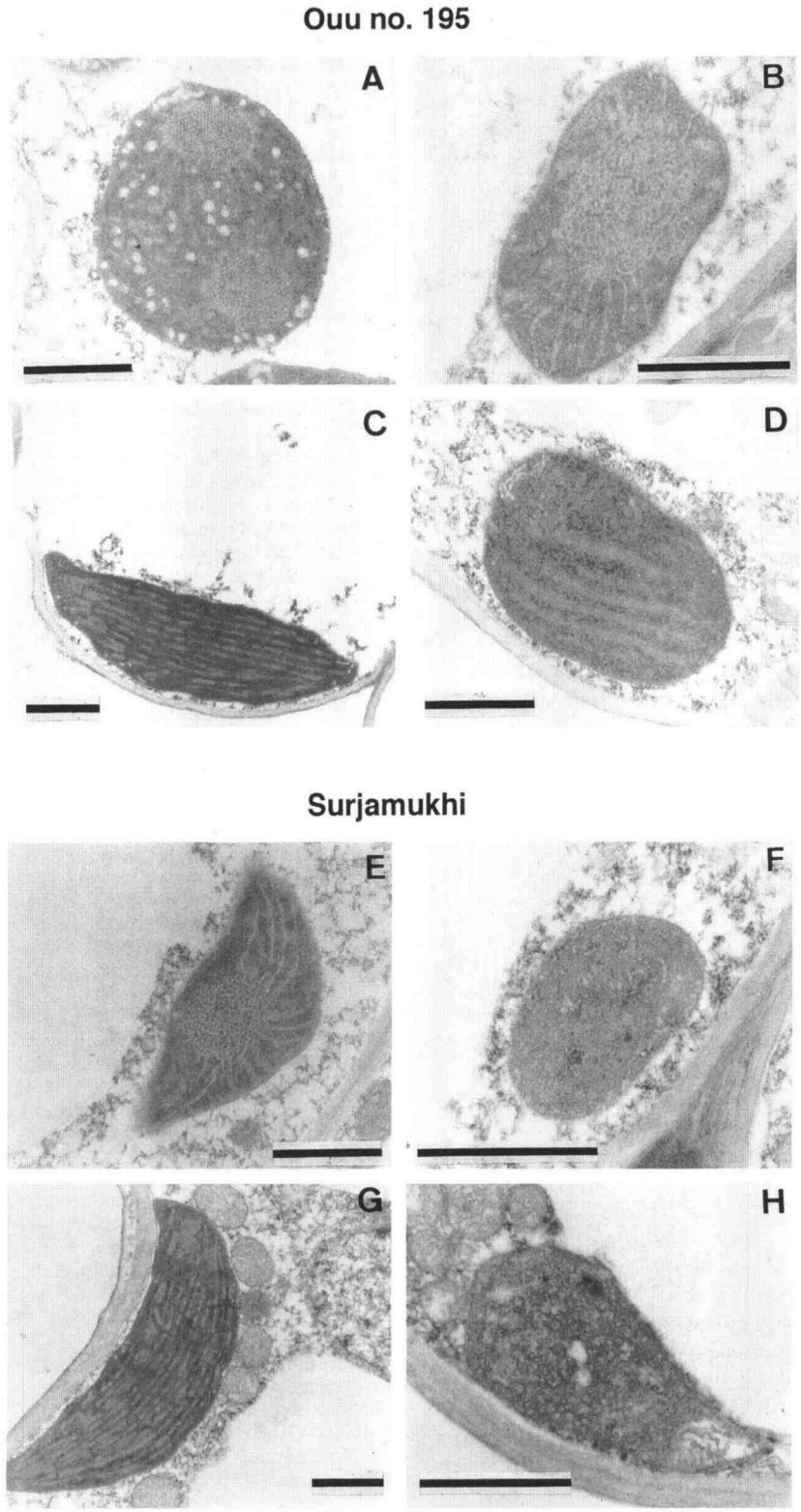

Figure 9. Ultrastructure of plastids in the second leaves excised from seedlings immediately after growth in darkness at 15°C and after transfer to light and warm conditions. A to D, O-195; E to H, SUR. A and D, Plastids in second-leaf cells after growth at 25°C in darkness. B and F, Plastids in second-leaf cells after growth at 15°C in darkness. C and G, Plastids in second-leaf cells after illumination for 24 h at 25°C subsequent to dark growth at 25°C. D and H, Plastids in second-leaf cells after illumination for 24 h subsequent to dark growth at 15°C. Details of EM are given in "Materials and Methods." Bars correspond to 1 μm.

development (Herrmann et al., 1992). Thus, chloroplast development requires both expression of intrinsic and light-dependent genes. Since, in our experimental system, CTIC is observed only in newly developing second leaves after transfer of dark-grown seedlings to light and warm conditions, the major cause of the induction of CTIC seems to be related to interference in the expression of light-independent, intrinsic genes, which are indispensable for the development of normal etioplasts.

Resembling other plastid proteins encoded by nuclear genes, the POR protein is synthesized as a larger precursor (pPOR) in the cytosol (Keegstra et al., 1989; Archer and Keegstra, 1990). The accumulation of the POR protein in plastids has been considered to be regulated by the rate of transcription, by posttranscriptional processing and modification, and by posttranslational protein assembly (Tobin and Silverthorne, 1985; Mullet, 1988; Thompson and White, 1991). Based on the evidence as reported, the decline in the POR level in the sensitive rice cultivar as a function of cool temperatures is also thought to be regulated at the posttranscriptional levels. Hess et al. (1992) reported that the protein level of POR in a dark-grown albina mutant of barley was markedly suppressed to 5 to 10% of that in the wild type, whereas the mRNA level is suppressed only to the 50% level of the wild type. In our preliminary experiments, no substantial differences were observed in the level of the transcript between different cultivars or between seedlings of the same cultivar at different growth temperatures in darkness (R. Yoshida, unpublished data). Recently, Reinbothe et al. (1995) provided evidence that the posttranslational processing and assembly of the POR precursor (pPOR) is regulated by the levels of the substrate (Pchlide) inside the plastids.

During differentiation and growth of leaf cells, the proplastids differentiate to chloroplasts in synchrony with cell growth (see refs. in Mullet, 1988). Dahlin and Cline (1991) also reported evidence to suggest that the accumulation of POR in plastids might be under developmental control.

Given that the growth of shoots and the development of immature leaves within the leaf sheath occur simultaneously, the noncoordinated progress between cell division or cell growth and the development of undifferentiated plastids to etioplasts, which may occur during growth within a narrow range of cool temperature, are considered to play an important role in the induction of CTIC in rice. According to Iba et al. (1991) and Kusumi et al. (1994), in a virescent mutant of rice, the virescent gene is thought to be expressed immediately after the growth stage in which leaf cells differentiate and generate the basic structure of leaf. Thus, the gene(s) controlling expression of CTIC might also be closely associated with the gene(s) that control the development of plastids in immature leaves.

ACKNOWLEDGMENT

We are grateful to Dr. Seizo Fujikawa (Institute of Low Temperature Science, Hokkaido University, Japan) for kindly teaching us the transmission EM technique.

Received October 5, 1995; accepted December 2, 1995.

LITERATURE CITED

Apel K (1981) The protochlorophyllide holochrome of barley (*Hordeum vulgare* L.). Phytochrome-induced decrease of translatable mRNA coding for the NADPH-protochlorophyllide oxidoreductase. Eur J Biochem **120:** 89–93

Archer EK, Keegstra K (1990) Current views on chloroplast protein import and hypotheses on the origin of the transport mechanism. J Bioenerg Biomembr **22:** 789–810

Beale SI, Weinstein JD (1990) Tetrapyrrole metabolism in photosynthetic organisms. *In* HA Dailey, ed, Biosynthesis of Heme and Chlorophylls. McGraw-Hill, New York, pp 287–391

Boffey SA, Ellis JR, Sellden G, Leech RM (1979) Chloroplast division and DNA synthesis in light-grown wheat leaves. Plant Physiol **64:** 502–505

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem **72:** 248–254

Choung PV, Omura T (1982) Studies on the chlorosis expressed under low temperature in rice, *Oryza sativa* L. *In* Bulletin of the Institute for Tropical Agriculture, Vol 5. Kyusyu University, Fukuoka, Japan, pp 1–58

Dahlin C, Cline K (1991) Developmental regulation of the plastid import apparatus. Plant Cell **3:** 1131–1140

Dehesh K, Cleve BV, Ryberg M, Apel KL (1986a) Light-induced changes in the distribution of the 36000-Mr polypeptide of NADPH-protochlorophyllide oxidoreductase within different cellular compartments of barley (*Hordeum vulgare* L.). II. Localization by immunogold labeling in ultrathin sections. Planta **169:** 172–183

Dehesh K, Klaas M, Häuser I, Apel K (1986b) Light-induced changes in the distribution of the 36000-Mr polypeptide of NADPH-protochlorophyllide oxidoreductase within different cellular compartments of barley (*Hordeum vulgare* L.). I. Localization by immunoblotting in isolated plastids and total leaf extracts. Planta **169:** 162–171

Dehesh K, Ryberg M (1985) The NADPH-protochlorophyllide oxidoreductase is the major protein constituent of prolamellar bodies in wheat (*Triticum aestivum* L.). Planta **164:** 396–399

Forreiter C, Apel K (1993) Light-independent and light-dependent protochlorophyllide-reducing activities and two distinct NADPH-protochlorophyllide oxidoreductase polypeptides in mountain pine (*Pinus mugo*). Planta **190:** 536–545

Harlow E, Lane D (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Häuser I, Dehesh K, Apel K (1987) Light-induced changes in the amounts of the 36000-Mr polypeptide of NADPH-protochlorophyllide oxidoreductase and its mRNA in barley plants grown under a diurnal light/dark cycle. Planta **170:** 453–460

He ZH, Li J, Sundqvist C, Timko MP (1994) Leaf developmental age controls expression of genes encoding enzymes of chlorophyll and heme biosynthesis in pea (*Pisum sativum* L.). Plant Physiol **106:** 537–546

Herrmann RG, Westhoff P, Link G (1992) Biogenesis of plastids in higher plants. *In* RG Herrmann, ed, Plant Gene Research: Cell Organelles. Springer-Verlag, New York, pp 276–332

Hess WR, Schendel R, Rüdiger W, Fieder B, Börner T (1992) Components of chlorophyll biosynthesis in a barley albina mutant unable to synthesize δ-aminolevulinic acid by utilizing the transfer RNA for glutamic acid. Planta **188:** 19–27

Hopkins WG (1982) Formation of chloroplast pigments in a temperature-sensitive, virescent mutant of maize. Can J Bot **60:** 737–740

Hopkins WG, Elfman B (1984) Temperature-induced chloroplast ribosome deficiency in virescent maize. J Hered **75:** 207–211

Hoshikawa K (1989) The growing rice plant. Nobunkyo, Tokyo, pp 28–29

Iba K, Takamiya K, Toh Y, Satoh H, Nishimura M (1991) Formation of functionally active chloroplasts is determined at a limited

stage of leaf development in virescent mutants of rice. Dev Genet **12:** 342–348

Kaimori N, Takahashi N (1985) Expression of chlorosis at low temperature in rice plants. Society for the Advancement of Breeding Researches in Asia and Oceania Journal **17:** 57–66

Keegstra K, Olsen LJ, Theg SM (1989) Chloroplastic precursors and their transport across the envelope membranes. Annu Rev Plant Physiol Plant Mol Biol **40:** 471–501

Kirk JTO, Tilney-Basset RAE (1978) The Plastids, Ed 2. Elsevier/North Holland Biomedical, Amsterdam, The Netherlands

Kusumi K, Inada H, Kawabata S, Iba K, Nishimura M (1994) Chlorophyll deficiency caused by a specific blockage of the C5-pathway in seedlings of virescent mutant rice. Plant Cell Physiol **35:** 445–449

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lyons JM (1973) Chilling injury in plants. Annu Rev Plant Physiol **24:** 445–466

Millerd A, McWilliam JR (1968) Studies on a maize mutant sensitive to low temperature. I. Influence of temperature and light on the production of chloroplast pigments. Plant Physiol **43:** 1967–1972

Moran R (1982) Formulae for determination of chlorophyllous pigments extracted with *N,N*-dimethylformamide. Plant Physiol **69:** 1376–1381

Moran R, Porath D (1980) Chlorophyll determination in intact tissues using *N,N*-dimethylformamide. Plant Physiol **65:** 478–479

Mullet J (1988) Chloroplast development and gene expression. Annu Rev Plant Physiol Plant Mol Biol **39:** 475–502

Nagamine T (1991) Genetic control of tolerance to chilling injury at seedling stage in rice, *Oryza sativa* L. Jpn J Breed **41:** 35–40

Reinbothe S, Runge S, Reinbothe C, Cleve BV, Apel K (1995) Substrate-dependent transport of the NADPH:protochlorophyllide oxidoreductase into isolated plastid. Plant Cell **7:** 161–172

Ryberg M, Sundqvist C (1982) Characterization of prolamellar bodies and prothylakoids fractionated from wheat etioplasts. Physiol Plant **56:** 125–132

Santel HJ, Apel K (1981) The protochlorophyllide holochrome of barley (*Hordeum vulgare* L.). The effect of light on the NADPH:protochlorophyllide oxidoreductase. Eur J Biochem **120:** 95–103

Schneider JC, Nielsen E, Somerville C (1995) A chilling-sensitive mutant of *Arabidopsis* is deficient in chloroplast protein accumulation at low temperature. Plant Cell Environ **18:** 23–32

Schultz R, Senger H (1993) Protochlorophyllide reductase: a key enzyme in the greening process. *In* C Sundqvist, M Ryberg, eds, Pigment-Protein Complexes in Plastids: Synthesis and Assembly. Academic Press, New York, pp 49–54

Schultz R, Steinmüller K, Klaas M, Forreiter C, Rasmussen S, Hiller C, Apel K (1989) Nucleotide sequence of a cDNA coding for the NADPH-protochlorophyllide oxidoreductase (PCR) of barley (*Hordeum vulgare* L.) and its expression in *Escherichia coli.* Mol Gen Genet **217:** 355–361

Spano AJ, He Z, Michel H, Hunt DF, Timko MP (1992) Molecular cloning, nuclear gene structure, and developmental expression of NADPH-protochlorophyllide oxidoreductase in pea (*Pisum sativum* L.). Plant Mol Biol **18:** 967–972

Susek RE, Ausubel FM, Chory J (1993) Signal transduction mutants of *Arabidopsis* uncouple nuclear *CAB* and *RBCS* gene expression from chloroplast development. Cell **74:** 787–799

Tam JP (1988) Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc Natl Acad Sci USA **85:** 5409–5413

Taylor WC (1989) Regulatory interactions between nuclear and plastid genomes. Annu Rev Plant Physiol Plant Mol Biol **40:** 211–233

Thompson WF, White MJ (1991) Physiological and molecular studies of light-regulated nuclear genes in higher plants. Annu Rev Plant Physiol Plant Mol Biol **42:** 423–466

Tobin EM, Silverthorne J (1985) Light regulation of gene expression in higher plants. Annu Rev Plant Physiol **36:** 569–593

Wray W, Boulikas T, Wray VP, Hanock R (1981) Silver staining of proteins in polyacrylamide gels. Anal Biochem **118:** 197–203