Short Communication

Chloroplast biogenesis during the early stage of leaf development in rice

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Abstract In rice, the developmental process in leaf formation can be divided into 7 stages (stages P0 to P6). We investigated chloroplast biogenesis and physiological changes in the developing leaves at the stage P4, during which leaf blade elongation and establishment of basic leaf blade structure occur. Chlorophyll content was negligible in the leaves early in the P4 stage and increased rapidly as they enters the late P4 phase. Chlorophyll fluorescence ratio (Fv/Fm) also increased markedly and the final value was comparable with that of mature leaves. Gene expression analysis showed that during the P4 stage, chloroplasts in the leaf cell undergo all three steps of differentiation: (i) plastid division and DNA replication (ii) establishment of plastid genetic system (iii) activation of photosynthetic apparatus. These observations suggest that the P4 is key in the development of a leaf, during which leaf rapidly differentiated both morphologically and physiologically, and that the P4 leaf is suitable for investigation of physiological relationships between chloroplast and leaf development.

Key words: Chloroplast biogenesis, *Oryza sativa*.

Chloroplast biogenesis during leaf development consists of a series of complex events. Based on the previous studies on both dicots and monocots, the events accompanying chloroplast differentiation can be divided into three steps (Mullet 1993). The first step involves the activation of plastid replication and plastid DNA synthesis. The second step is the chloroplast "build-up" step, which is characterized by the establishment of the chloroplast genetic system. At this step nuclear-encoded plastid RNA polymerase, termed NEP, preferentially transcribes plastid genes encoding plastidal gene expression machineries (Hajdukiewicz et al. 1997), and the transcription and translation activity in the chloroplast is dramatically increased. In the final step, the plastid and nuclear genes encoding photosynthetic apparatus are expressed at very high levels. Plastid genes for photosynthetic apparatus are mainly transcribed by plastid-encoded RNA polymerase, termed PEP (De Santis-MacIossek et al. 1999). Expressions of these genes lead to the synthesis and assembly of the photosynthetic machineries accompanied with the activation of photosynthetic competence.

Developing chloroplasts are photosynthetically immature, but they are presumed to develop other sub-functions in response to the leaf cell differentiation. For example, during ontogeny, leaves undergo transition from net carbon importers (sink) to net carbon exporters (source) (Turgeon 1989). It is highly possible that chloroplast has a central role in the internal regulation of carbon assimilation, storage and distribution, which strongly correlates with the sink-source control. However, details of the physiological relationship between developing chloroplast and host leaf cells during early leaf development are still poorly understood. In this study, we investigated events of chloroplast biogenesis mentioned above during early leaf development in rice to obtain basic information for understanding interactions between chloroplast and leaf development.

Rice plants are known to show the striking feature of leaf primordia production (plastochron) being closely synchronized with the leaf emergence called phyllochron (Nemoto and Yamazaki 1993; Itoh et al. 2005) in shoot development. This enables the definition of a series of successive stages of leaf development, starting with P0 (leaf founder), through P1 (youngest primordium), P2, P3, P4, P5 and P6 (fully expanded leaf) (Figure 1A). Among these stages, leaf morphology changes most drastically during the P4 stage. Anatomical studies

Abbreviations: Chl, chlorophylls; NEP, nuclear-encoded plastid RNA polymerase; PEP, plastid-encoded plastid RNA polymerase
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showed that in rice the P4 stage is characterized by the rapid leaf blade elongation (Itoh et al. 2005). Differentiation of epidermal specific cells, such as stomata, also occurs at this stage. When leaves begin to emerge, leaf tissues are visibly green, whereas those corresponding to early P4 are pure white (Figure 1B). Furthermore, our previous studies showed that expressions of NEP and plastid genes for plastid transcription and translation apparatus occur in the leaves at this stage (Kusumi et al. 2004; Sugimoto et al. 2007), suggesting that early processes of chloroplast differentiation are expected to occur during the P4 stage.

In this study, we used rice seedlings with fully emerged third leaves. In these seedlings, the fourth leaves correspond to the P4 stage (P4 leaves) (Figure 1A). For preparing tissue samples, seeds of *Oryza sativa* L. cv. Taichung 65 were sown on a commercial soil mixture (Baiyodo; SunAgro, Japan) in plastic pots (diameter, 120 mm; depth, 100 mm), and placed in a growth chamber (Advantec IS-2300) maintained at a temperature of 28°C with constant fluorescent lighting (200 μmol m^{-2}). The 8- to 10-day-old seedlings were collected and fourth leaves were extracted from inside the third leaf sheath. Under these growth conditions, P4 leaves had an initial length of 3-5 mm and reached a final size of about 10 cm (Figure 1B). For precise characterization of the P4 stage, we divided it into several substages designated as P4-X. X indicates the leaf length. For example, leaves shorter than 1 cm are involved in the stage P4-1. One to 2 cm leaves belong to the P4-2. In the rice seedlings we used in this study, P4-10 is the final substage that includes 9 to 10 cm leaves. Figure 1C shows the chlorophyll content in the P4 leaves. Leaf tissues were homogenized and the pigments were extracted from the homogenate with 80% acetone under dim light (Kusumi et al. 2000). Chl content was determined using the Lichtenthaler equation (1987). Chl concentration per unit fresh weight was negligible in the early P4 stage, and increased almost linearly with the increase in leaf length. At the P4-10 stage, chlorophyll content in the leaf reached about 40% of that in a mature P5 leaf.

Chlorophyll fluorescence parameter Fv/Fm indicates the maximum quantum yield of PSII photochemistry and is tightly related to the physiological state of chloroplast (Krause and Weiss 1991; Baker 2008). We captured Chl fluorescence images of detached P4 leaves by using the imaging fluorometer (FluorCam 701MF, Photon System Instruments, Czech Republic). Images of F_0 and Fm were obtained separately using CCD camera. These images were subtracted by FluorCam software (ver 6; Photon System Instruments) and divided [(Fm$-F_0$)/ Fm] to generate an image of Fv/Fm (Figure 2A). Chl fluorescence was undetectable in the P4-1 leaves (data not shown). From P4-2 to P4-10, the mean Fv/Fm values for the whole leaf substantially increased from 0.52 to 0.64 (Figure 2A). The monocot leaf grows in length from a meristem at its base. Therefore, meristematic cells on the leaf base contain undeveloped plastids. More differentiated cells containing more developed chloroplasts are located close to the leaf tip (Kusumi et al. 1997). Chl fluorescent images showed similar spatial patterns in the Fv/Fm value gradually increased in the leaf tips from the base portion (Figure 2B). Even in the P4-4 leaf, Fv/Fm value at the leaf tip was above 0.7, which is comparable to the value of mature leaves. At the P4-10 stage, the Fv/Fm in the upper half of the leaf was above 0.7, while that in the basal portion was below 0.5.

We next examined the changes in chloroplast ultrastructure in the developing P4 leaves by electron microscopy. Small pieces of the tissue detached from middle portion of each leaf were fixed in the primary fixation buffer (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4). They were then rinsed three times in 0.1 M cacodylate buffer, pH 7.4, and fixed with secondary fixation buffer (1% OsO4 in 0.1 M cacodylate buffer, pH 7.4). The samples were dehydrated in a graded series of ethanol dilutions (30, 50, 70, 90 and 100%) and embedded in an epon resin mixture (TAAB Epon 812, TAAB Laboratories Equipment Ltd., Berkshire, UK). Ultra-thin sections were stained with 2% (w/v) aqueous uranyl acetate for observation (Kusumi et al. 2000). Chloroplasts observed in the P4-1 leaf were characteristic and distinct from those observed at later stages (Figure 3A). They are sub-spherical with the diameter of about 1 μm or more. Internal membranes are almost absent, and the starch grains are present instead. Low Chl content (Figure 1) and low PSII activity (Figure 2) in the P4-1 leaves indicate that these chloroplasts have little photosynthetic activity. These starch grains were presumed to have been converted from sugars translocated from the upper (older) leaf, and these chloroplasts temporarily function as storage organelles, like amyloplasts. The chloroplasts in the P4-2 leaves became larger and contained several internal membrane structures. Starch grains were absent (Figure 3B). During middle to later stages (P4-4 to P4-10), the number and size of chloroplasts gradually increased along with the development of the thylakoid membrane system (Figure 3C-F). Overall, the structure of chloroplasts developed continuously during the P4 stage. We could not find distinct, stage-specific structure occurring in the chloroplasts except for the starch grain observed in the leaves at the P4-1 stage.

To monitor the degree of chloroplast development at the molecular level, we examined the transcript accumulations of nucleus-encoded (*OsPOLP1*, *FtsZ*, *OsRpoTp*, *V2*, *Lhcb* and *rbcS*) and plastid-encoded (*trnE*, *rpoA*, *rbcL* and *psbA*) genes in the leaves at the P4 stage. *OsPOLP1* and *FtsZ* encode plastidal DNA polymerase

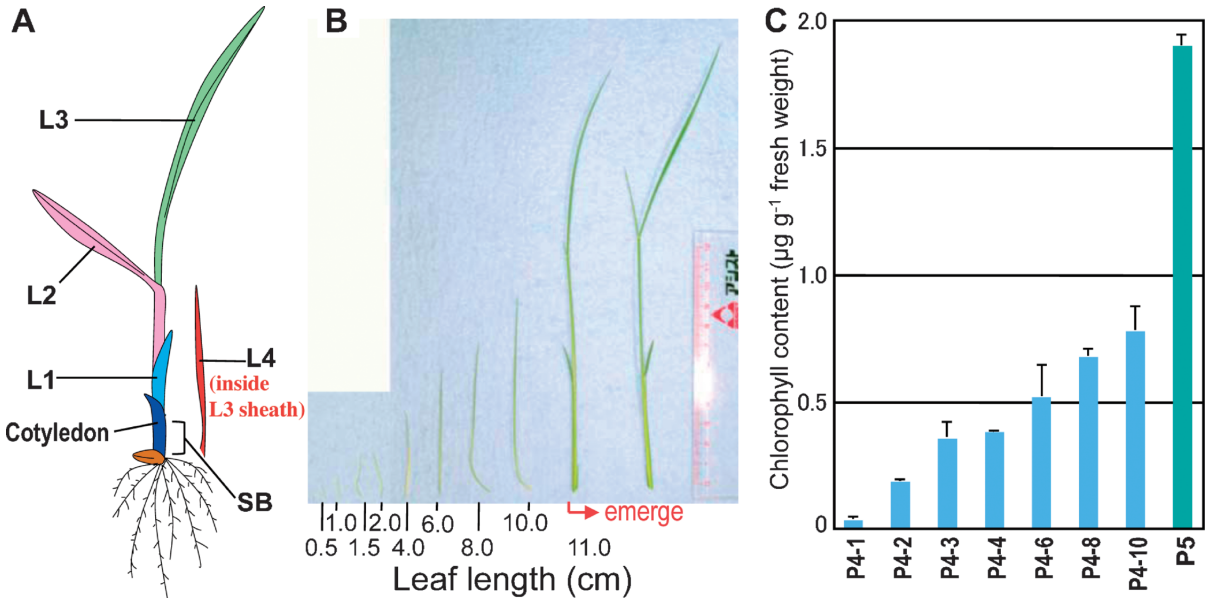

Figure 1. The fourth leaf at the developmental stage P4. (A) Schematic illustration of a rice seedling with fully expanded third leaf. L1, L2, L3 and L4 indicate the first, second, third and forth leaf, respectively. SB (shoot base) corresponds to a 5 mm piece from the bottom of the shoot. (B) Photograph of the fourth leaves inside L3 sheath. The leaf size is given below each photograph. (C) Total chlorophyll content in the developing L4 leaves. The size of the leaf used is given below each bar. Error bars show SD of three experiments.

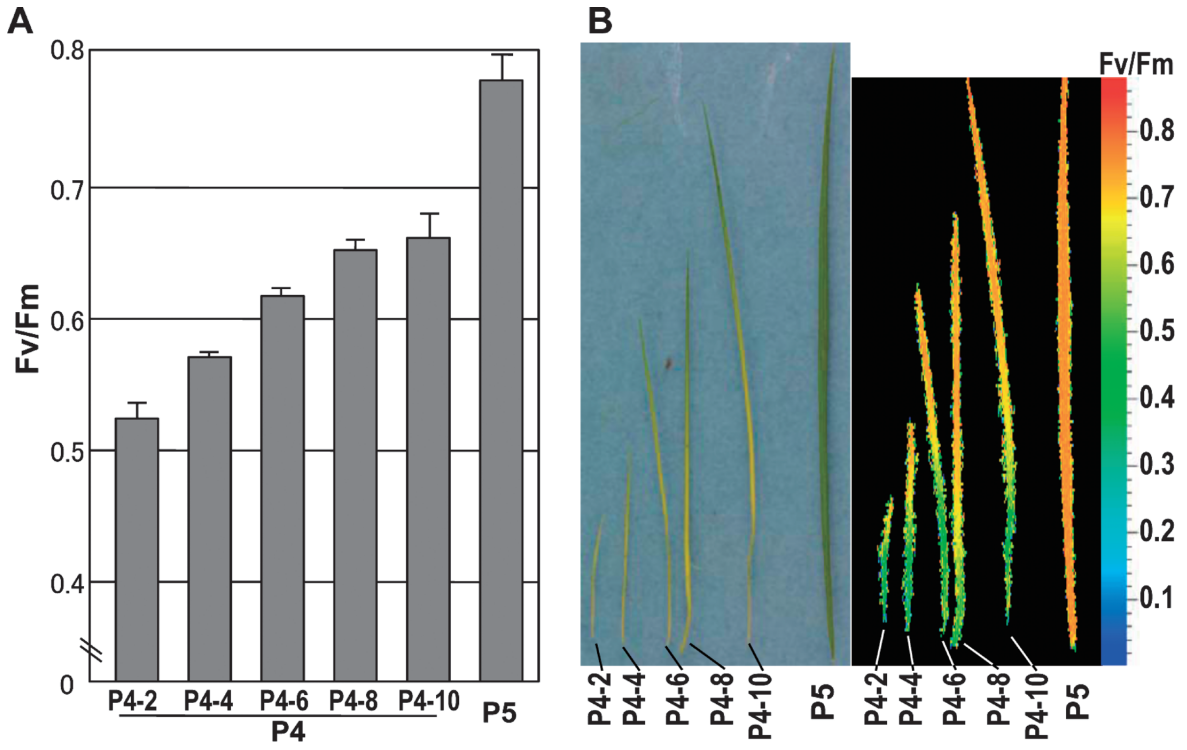

Figure 2. Chlorophyll fluorescent in the developing leaves at P4 stage. All data were obtained using the FluorCam imaging system as described in the text. (A) Mean Fv/Fm value in whole leaves. Values represent mean±SD for whole leaves indicated in (B). (B) Photograph of developing leaves at P4 and P5 stages (left) and a representative false color image of Fv/Fm (right). Fv/Fm values are represented between the blue (0, lowest) and red (1.0, highest) extremes of the false color scale.

and a component of plastid division machinery respectively (Vitha et al. 2001; Takeuchi et al. 2007). Their expression is involved in the first step of chloroplast differentiation. Os*RpoTp*, *V2* and *rpoA* encode NEP, plastidal guanylate kinase and PEP α subunit respectively (Hiratsuka et al. 1989; Kusumi et al. 2004; Sugimoto et al. 2007). They are involved in the regulation of chloroplast transcription/translation

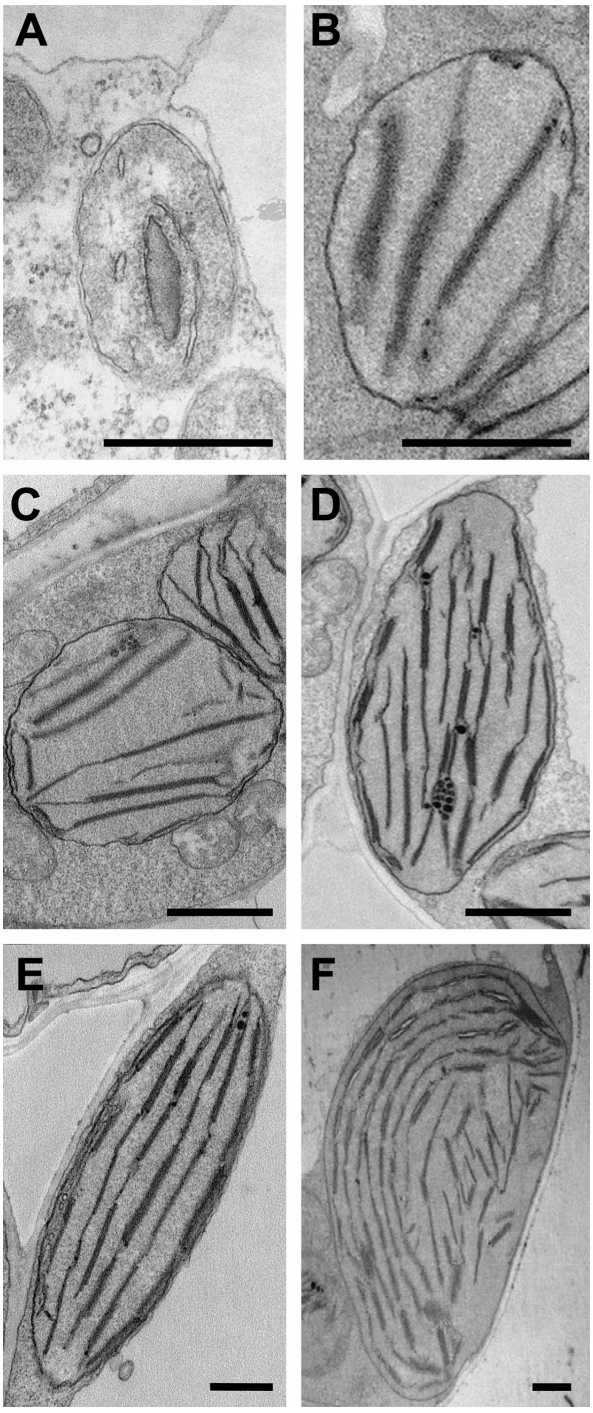

Figure 3. Electron micrographs of chloroplasts in the developing leaves at the P4 stage. Tissues were collected from middle portions of the leaf at the developmental stage P4-1 (A), P4-2 (B), P4-4 (C), P4-8 (E) and P5 (F), or from the tip portions of the leaf at the stage P4-4 (D). Bars = 1 μm.

activity, and highly transcribed at the second step (Kusumi et al. 2004; Sugimoto et al. 2007). *Lhcb*, *rbcS*, *rbcL* and *psbA* encode photosystem II (PSII)-associated light-harvesting chlorophyll protein, small and large subunit of ribulose-1,5-bisphosphate carboxylase and the D1 subunit of PSII complex respectively (Hirai et al. 1985; Matsuoka 1990). They function in the regulation of photosynthetic apparatus that occur in the third step of chloroplast differentiation. *trnE* encodes $tRNA^{Glu}$, a multifunctional molecule required not only for translation but also for the chlorophyll biosynthesis and NEP repression (Schön et al. 1986; Hanaoka et al. 2005). In addition to the L4 and L3 leaf blade, we used a 5 mm piece from the bottom of the shoot (SB) (Figure 1A) for RNA preparation. In the rice seedlings with a fully emerged third leaf, the part SB contains the seventh to the fifth immature leaves at the P0 to P3 stages. RNA isolation, RT-PCR reactions and RNA-blot analysis were carried out as previously described (Kusumi et al. 2004; Sugimoto et al. 2007). The sequences of gene-specific primers used for RT-PCR reactions are as follows: *OsPOLP1*, 5′-ACCGGTGCTTTCAGGCTTGG-3′ and 5′-GCTGACTGATAATCACACG-3′; *FtsZ*, 5′-AAAGG-ACATAACCTTGCAAG-3′ and 5′-AGTTTTCCTATTG-AACCGTG-3′; *OsRpoTp*, 5′-AAGCAGACAGTGATG-ACATC-3′ and 5′-ATCACATGCATGCACCCAAA-3′; V2, 5′-GAGGAGTTCCTCACGATGAT-3′ and 5′-CAG-CATCATGATAGACTCC-3′; *Lhcb*, 5′-CCGTCAACAA-CAACGCCTGG-3′ and 5′-ACATCATCTTCTTCTT-CATC-3′; *rbcS*, 5′-CTAACTAACTACGTGCTATG-3′ and 5′-CGATGCTTGATCTTAGCTTA-3′. The DNA probes for *trnE*, *rpoA* and *rbcL* used for the Northern analysis were prepared as described (Kusumi et al. 2004).

Transcripts for genes that function in the first step of chloroplast differentiation (*OsPOLP1*, *FtsZ*) are already present in the P0 to P3 stages (Figure 4A), and decrease gradually during the P4 stage. Transcripts for *OsRpoTp*, *V2* and *rpoA* reached maximal abundance in the leaves at stages P4-2 and P4-4 and dropped down while the transcript levels coding for *Lhcb*, *rbcS*, *rbcL* and *psbA* augmented continuously latter in the P4 stage (Figure 4A and 4B). $tRNA^{Glu}$ encoded by *trnE* increased from P4-2 to P4-8 was repressed at P4-10, and increased again at the P5 stage after leaves start to emerge. These observations suggest that the first step of chloroplast differentiation is likely to start in the leaves at the P0 to P3 stages, and will largely finish during early P4 stage (Figure 4C, I). The second stage occurs significantly in the leaves at around P4-2 and P4-4, and the decline of the second step and onset of the third step take place during P4-6 to P4-8 (Figure 4C, II and III). This transition coincides with the first activation of $tRNA^{Glu}$, which is reported to mediate NEP/PEP switching (Hanaoka et al. 2005). Plastid genes involved in the second step are known to be mainly transcribed by NEP, and those involved in the third step are transcribed by PEP (Hajdukiewicz et al. 1997; De Santis-MacIossek et al. 1999). Therefore, activation of *trnE* during this phase could correlate with the requirement of $tRNA^{Glu}$ for the NEP-PEP switching.

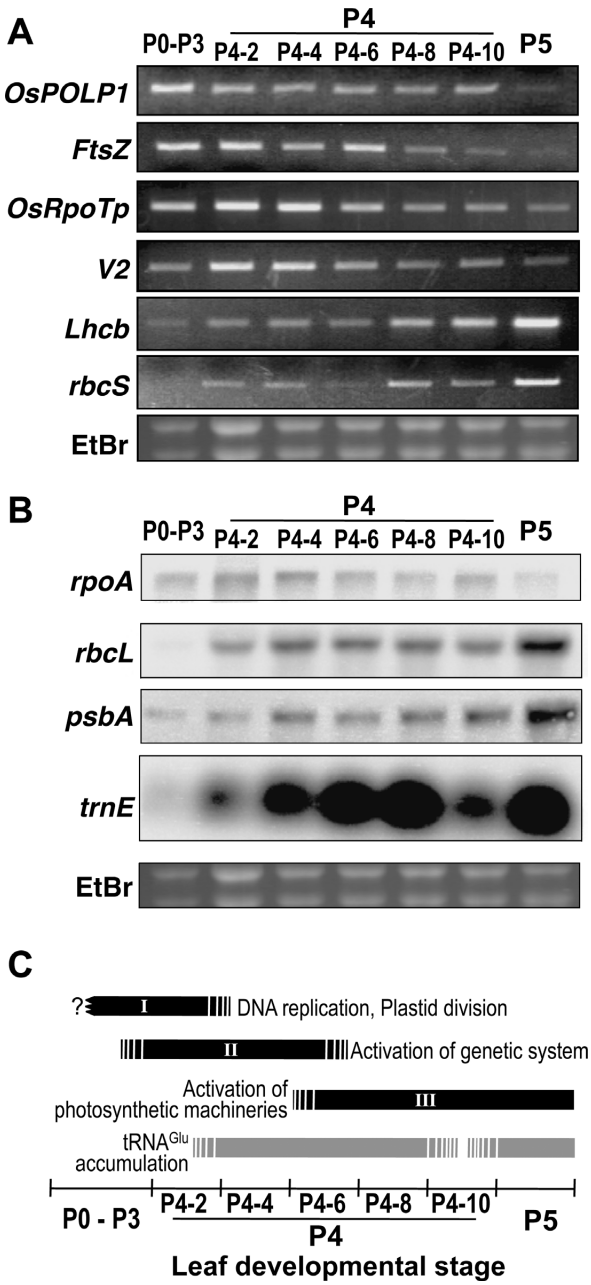

Figure 4. Analysis of mRNA accumulation of chloroplast proteins during the P4 stage. (A) mRNA levels of nuclear genes encoding chloroplast proteins, *OsPOLP1*, *FtsZ*, *RpoTp*, *V2*, *Lhcb* and *rbcS*, monitored by RT-PCR. (B) Northern blot analysis of plastid-encoded genes. Ten μg of total RNA were loaded per lane, and hybridized with probes for the genes *rpoA*, *rbcL*, *psbA* and *trnE*. (C) Schematic representation of the chronological progression of principal growth steps during leaf development. Horizonal bars indicate the period when the indicated events occur in the chloroplast.

In conclusion, our observations suggest that the major part of early chloroplast development, from plastid replication to activation of photosynthetic machineries, occurs in the developing leaves at the P4 stage. Three steps of chloroplast differentiation progress in an ordered manner, and they could be distinguished easily by using molecular probes and markers (Figure 4). Since adult leaves follow a similar developmental manner in rice (Nemoto and Yamazaki 1993; Itoh et al. 2005), our results will be applicable to the seedlings of different ages. On the other hand, in *Arabidopsis*, intensive activation of plastidal transcription and translation apparatus has been observed only during germination and early seedling development (Harrak et al. 1995; Hanaoka et al. 2005; Demarsy et al. 2006), suggesting that it is difficult to identify the foliage leaf tissue that contains chloroplasts at particular developmental step. P4 leaves of rice will be an attractive research material suitable for studying regulation of chloroplast biogenesis during early leaf development.

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