Changes in Levels of Thylakoid Components in Chloroplasts of Pine Needles of Different Ages

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Levels of thylakoid components in chloroplasts and transcripts of photosynthesis-related genes were compared in leaves of different ages, namely, current, 1-year-old and 2-year-old needles, of a Japanese black pine tree (Pinus thunbergii). Levels of thylakoid components, PSI, PSII and Cyt b6/f complexes, measured as P700, Cyt b599 and Cyt f, respectively, were lowest in current needles, on a per needle basis, and were almost eight times higher in 1-year-old needles. Levels of PSI and Cyt b6/f complexes were slightly lower in 2-year-old needles while the level of PSII remained unaltered or was even slightly higher than in 1-year-old needles. The ratio of PSI to PSII decreased markedly as needles aged. By contrast, levels of cab, psaA/B, psbA, rbcS and rbcL transcripts were the highest in current needles, and the levels decreased with increasing age. The extent of the reduction was the greatest for psaA/B transcripts, with the level in 2-year-old needles being about 30% of that in current needles. The smaller reduction was observed in the level of psbA transcripts with about 90% of the current-year level in 2-year-old needles. These results suggest that PSII complex accumulates more slowly than PSI complex during leaf development, and that PSI and Cyt b6/f complexes decay before PSII during the initial period of senescence.

Key words: Japanese black pine (Pinus thunbergii) — Levels of transcripts — Needles of different ages — Photosynthesis-related genes — Ratio of PSI to PSII — Thylakoid components.

The recent recognition of the fact that forests play an important role in the maintenance of the global environment has accelerated ecophysiological studies of woody plants, with emphasis on their responses to environmental stress (Troleng and Linder 1982, Dietz et al. 1988, Tan et al. 1992, Mousseau and Saugier 1992, Ottander et al. 1995). We have studied on photosynthetic apparatus of coniferous plants, but our previous studies were limited to the development of chloroplasts in pine cotyledons (Yamamoto et al. 1991, Shinohara et al. 1992a, b, 1994). We have not yet examined the dynamic features of chloroplasts in leaves of adult trees.

As a first approach to understanding the dynamics of chloroplast development in leaves of adult trees, in this study, we compared levels of thylakoid electron-transport components and transcripts of genes for chloroplast proteins in leaves (needles) of different ages of a pine tree. Leaves of Japanese black pine (Pinus thunbergii) survive for two years and a half. Leaf buds sprout in springtime and reach maturity in the autumn of the first year. Mature leaves remain alive for a further two years with a gradual decrease in photosynthetic activity (Clark 1961, Kramer and Kozlowski 1979). In this study, we compared levels of components of chloroplasts in leaves in the early summer of the first, the second and the third year. Our results indicate that (1) leaf expansion in the first year is accompanied by active development of chloroplasts with synthesis of chloroplast proteins de novo, (2) chloroplast components are maintained in a functional state for more than two years, and (3) chloroplast senescence starts early in the third year.

Materials and Methods

Plant material—Branches of a 30-year-old Japanese black pine tree (Pinus thunbergii) grown in the field of the Forestry and Forest Products Research Institute (36°00'N, 140°09'E), were collected at noontime at the beginning of July, 1988. Current, 1-year-old and 2-year-old needles (Fig. 1) were harvested from branches and stored at −80°C until use.

Preparation of thylakoid membranes—Frozen needles (10 g fresh weight) were powdered with a mortar and pestle in liquid nitrogen. Powdered needles were homogenized with a Polytron in 50 ml of 50 mM HEPES-KOH (pH 7.6), 10 mM EDTA, and 10% (w/v) PEG-4000. Homogenates were filtered through two layers of Miracloth. The debris was resuspended in the same buffer, homogenized again, and filtered. Combined filtrates were centrifuged at 20,000 x g for 60 min. Pellets were suspended in 5 ml of 50 mM HEPES-KOH (pH 7.6) and 10 mM EDTA. Each suspension was layered onto a three-step sucrose gradient (8 ml of 2 M sucrose, 15 ml of 1.3 M sucrose, and 8 ml of 0.4 M sucrose) in 50 mM HEPES-KOH (pH 7.6) plus 10 mM EDTA and centrifuged at 80,000 x g for 60 min. Thylakoid membranes were collected from the interface between 1.3 M and 2 M sucrose. They were diluted...
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Fig. 1 Current, 1-year-old and 2-year-old pine needles used in this study. A pine branch (A) and harvested pine needles (B) are shown. Leaf senescence begins in 2-year-old needles. The scale bars represent 2.0 cm.

about 6-fold with distilled water and centrifuged again at 20,000 × g for 60 min. The resultant pellets were stored at −80°C prior to analysis. All procedures were performed at 4°C under a dim green light.

Analysis of protein composition—For electrophoretic analysis of polypeptides in thylakoid membranes, stored membranes were suspended in 10 mM Na-PP₇ (pH 7.3) and washed three times with the same medium. Washed membranes were solubilized in SDS sample buffer (Laemmli 1970) at room temperature and subjected to SDS-PAGE on a slab gel of 13% (w/v) acrylamide containing 0.1% (w/v) SDS, in the discontinuous buffer system of Laemmli (1970). Proteins on gels were stained with Coomassie brilliant blue R-250.

Determination of the abundance of thylakoid components—The abundance of P700, Cyt b₅₅₉ and Cyt f in thylakoid membranes was determined spectrophotometrically using a Hitachi 557
spectrophotometer as described previously (Fujita and Murakami 1987, Shinohara et al. 1992a). After suspension in 50 mM Tricine-NaOH (pH 7.5), membranes were treated with 10 mM ferricyanide for 10 min at 4°C and used for quantitation of each component. The abundance of each component was calculated from the absorption difference coefficients of Hiyama and Ke (1972) for P700, of Böhme et al. (1980) for Cyt f and of Garewal and Wasser- 

man (1974) for Cyt b₅₉₃. Amounts of PSI and Cyt b₅₉₃ complexes were estimated as being equimolar to those of P700 and Cyt f. Levels of PSII were calculated from a stoichiometry of two Cyt b₅₉₃ per PSI, as described previously (Shinohara et al. 1992b). The amount of Chl and ratio of Chl a/b were determined spectrophotometrically with acetone extracts (80%) as described by Arnon (1949). Total protein was quantitated by the method of Lowry et al. (1951).

Isolation of RNA and Northern blot hybridization—Total RNA was prepared from current, 1-year-old and 2-year-old needles by the method of Chang et al. (1993) with slight modifications. In brief, frozen needles (2 g fresh weight) were powdered with a mortar and pestle in liquid nitrogen. The powdered needles were mixed well with 15 ml of a solution of 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% (w/v) hexadecyltrimethylammonium bromide, 2% (w/v) polyvinylpyrrolidone, 8.0), 25 mM EDTA, 2 M NaCl, 2% (w/v) hexadecyltrimethylammonium bromide, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) β-mercaptoethanol and 0.5 mg liter⁻¹ spermidine and kept at 65°C for 10 min. The solution was extracted with an equal volume of a mixture of chloroform and isooamyl alcohol (24 : 1, v/v) and centrifuged at 15,000 x g for 10 min. The above step was repeated two more times. One-fourth volume of 10 M LiCl was added to the final aqueous phase. Total RNA was precipitated overnight at 4°C and collected by centrifugation at 15,000 x g for 20 min. The pellet RNA was dissolved into 500 μl of a solution of 10 mM Tris-HCl (pH 8.0), 1 M NaCl, 0.5% (w/v) SDS, and the solution was extracted twice with a mixture of chloroform and isoamyl alcohol. After precipitation in ethanol, RNA was dissolved in sterile water and stored at −80°C prior to analysis.

For Northern blot hybridization, 10 μg of total RNA were fractionated by formaldehyde gel electrophoresis (Yamamoto et al. 1991). Separated bands of RNA were detected by staining with ethidium bromide and blotted on a nylon membrane. Methods for DNA labeling and hybridization were the same as those described previously (Mukai et al. 1991). Northern probes for cab and rbcL were 0.9-kb cDNAs from pine (Yamamoto et al. 1991). Probes for psbA, psaA/B and rbcL were 0.2-kb Pest-Pvull and 2.3-kb BamHI-BamHI internal DNA fragments of tobacco psbA and psaA/B (Shinozaki et al. 1988), and a 1.1-kb Smal-HindIII fragment of pine rbcL (Mukai et al. 1991), respectively. For quantitative determinations of transcripts, the intensities of corresponding bands on autoradiograms were monitored by scanning the X-ray film at 600 nm with a Shimadzu dual-wavelength flying-spot scanner (CS-9000).

Results and Discussion

Levels of thylakoid components in pine needles of three different ages—As shown in Figure 1, the green color of current needles appeared to be less intense than that of mature needles. The level of Chl in current needles, on a per needle basis, was almost one-eighth of that in mature needles (Table 1). The Chl content was highest in 1-year-old needles, and it was slightly lower in 2-year-old needles. The ratio of Chl a/b was the highest in current needles and it decreased slightly as the needles aged. A rapid increase in the level of Chl b during the first year indicated that a rapid increase in levels of light-harvesting Chl a/b proteins, most likely LHCII, accompanied the increase in levels of photosystem complexes.

The levels of protein in membranes prepared from current, 1-year-old and 2-year-old needles were 16.4, 13.0 and 11.0 mg (mg Chl)⁻¹, respectively. The protein content of thylakoid membranes, on a per needle basis, was the lowest in current needles and was six-fold higher in 1-year-old needles. The level was slightly (30%) lower in 2-year-old than in 1-year-old needles. Thus, the levels of Chl and protein in thylakoid membranes changed dramatically during leaf maturation in the first year. However, changes in the composition of thylakoid membrane proteins were not marked, with the exception of increases in the levels of 50.1-, 23.5- and 20.5-kDa polypeptides with increasing age (Fig. 2).

As suggested by the Chl content, a marked increase in levels of PSI and PSII complexes, indicated by increases in levels of P700 and Cyt b₅₉₃, respectively, occurred during the first year (Table 2). The amount of Cyt b₅₉₃ complex, in-

<table>
<thead>
<tr>
<th>Needle</th>
<th>Length (cm)</th>
<th>Fresh weight (mg)</th>
<th>Chl content</th>
<th>Ratio of Chl a/b</th>
<th>Recovered RNA (μg (g FW)⁻¹)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Chl a (mg (g FW)⁻¹)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Chl b (mg (g FW)⁻¹)</td>
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<td></td>
<td></td>
<td></td>
<td>Total</td>
<td></td>
<td></td>
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<tr>
<td>Current</td>
<td>4.28 ± 0.06</td>
<td>38.2</td>
<td>0.42 ± 0.01 (16)</td>
<td>0.14 ± 0.01 (5)</td>
<td>0.56 ± 0.02 (21)</td>
</tr>
<tr>
<td>1-year-old</td>
<td>11.17 ± 0.10</td>
<td>119.4</td>
<td>1.09 ± 0.02 (130)</td>
<td>0.38 ± 0.01 (45)</td>
<td>1.47 ± 0.03 (176)</td>
</tr>
<tr>
<td>2-year-old</td>
<td>9.73 ± 0.09</td>
<td>97.1</td>
<td>1.06 ± 0.04 (103)</td>
<td>0.39 ± 0.02 (38)</td>
<td>1.44 ± 0.06 (140)</td>
</tr>
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</table>

Table 1 Sizes and Chl contents of pine needles of three different ages, and recovery of RNAs from the needles

Over one hundred needles were used for determinations of the length and average fresh weight of a needle. Values of Chl content and recovered RNA are means ± SE of three measurements and means ± SE of results from four replicate experiments, respectively. Numbers in parentheses indicate average abundance (μg) on a per needle basis.
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C

Fig. 2 Composition of thylakoid membrane proteins in pine needles of different ages. Solubilized thylakoid membranes (2.5 µg Chl) from current (C), 1-year-old (1) and 2-year-old (2) pine needles were subjected to SDS-PAGE. Levels of three polypeptides (50.1, 23.5 and 20.5 kDa) indicated by arrowheads increased as leaves aged.

dicated by the level of Cyt f, also increased in parallel with increases in amounts of the two photosystem complexes. The pattern suggests that chloroplasts developed actively during the first year. The increase in protein content during leaf maturation support this suggestion. The increase in PSII content was more marked than that in PSI or Cyt b/f and, thus, the ratio of PSI to PSII decreased during the first year. A significant shift in the ratio of Chl a/b to smaller values during the first year (Table 1) suggests that the level of the Chl a/b protein complex, probably LHCII, increased more markedly than the level of Chl a protein complexes. Since the level of Chl a content in the PSI complex is far greater than that in the PSII complex (Mimuro and Fujita 1977, Myers et al. 1980, Fujita and Murakami 1987), the increase in Chl a protein complexes corresponds approximately to that in the PSI. This feature suggests that development of the PSII-associated apparatus is more marked than that of PSI during leaf development in the first year.

Levels of PSI and Cyt b/f complexes decreased slightly in 2-year-old needles while the level of PSII increased somewhat or remained at the level in 1-year-old needles (Table 2). The Chl b content on a fresh weight basis was also slightly higher in 2-year-old needles (Table 1). The level of LHCII probably also changes in a similar way to that of PSII. It is likely that the pattern of changes in the level of the PSII-associated apparatus is again different from that of PSI.

Levels of transcripts of genes for chloroplast proteins — The level of total RNA, on a fresh weight basis, was the highest in current needles and was dramatically lower in the 1-year-old needles (Table 1). The level was still lower in the 2-year-old needles. Since the majority of total RNA is rRNA, the level of rRNA must change in parallel with changes in total RNA. Levels of transcripts of genes for chloroplast proteins, namely, psaA/B, psbA and rbcL in the chloroplast genome and cab and rbcS in the nuclear genome, were determined by Northern blot analysis (Fig. 3). Levels of transcripts for cab (1.1 kb) and rbcS (0.95 kb) on a total RNA basis, were almost constant in leaves of all three different ages (Fig. 4A). However, levels of transcripts of psbA (1.25 kb) and rbcL (1.7 kb) increased as leaves aged. The increase was most prominent in the transcript of psbA. Conversely, the level of the psaA/B transcripts (5.0 kb) decreased markedly as leaves aged. A similar increase in transcripts of psbA and rbcL, and a similar decrease in transcripts of psaA/B were observed during the maturation of chloroplasts of barley seedlings (Klein and Mullet 1987, Kim et al. 1993). Transcripts of

<table>
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<tr>
<th>Source of thylakoid membranes</th>
<th>P700 (mmol (mol Chl)⁻¹)</th>
<th>Cyt f (mmol (mol Chl)⁻¹)</th>
<th>Cyt b₅₅₉ (mmol (mol Chl)⁻¹)</th>
<th>Ratio of PSI/PSII (mol (mol⁻¹))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current needles</td>
<td>2.10±0.14 (0.05)</td>
<td>2.21±0.08 (0.05)</td>
<td>2.06±0.34 (0.05)</td>
<td>2.04</td>
</tr>
<tr>
<td>1-year-old needles</td>
<td>1.72±0.10 (0.33)</td>
<td>2.01±0.07 (0.39)</td>
<td>2.83±0.04 (0.55)</td>
<td>1.22</td>
</tr>
<tr>
<td>2-year-old needles</td>
<td>1.59±0.05 (0.25)</td>
<td>1.54±0.09 (0.24)</td>
<td>4.18±0.67 (0.65)</td>
<td>0.76</td>
</tr>
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Values are means±SE of results from three replicate experiments. Numbers in parentheses indicate average abundance (nmol) on a per needle basis.
psaA/B were detected as RNAs of 5.0 kb and smaller, as in previous studies of transcripts of the psaA operon in tobacco chloroplasts (Meng et al. 1988). Amounts of smaller fragments (of around 1.5 kb) appeared to increase with decreases in levels of the 5.0-kb transcript. Since smaller fragments were probably degradation products of the 5.0-kb transcript, the pattern suggests that degradation of transcripts of psaA/B was accelerated as leaves aged.

The amount of total RNA on a per needle basis in 1-year-old needles was 80% of the amount in current needles (Table 1). Thus, levels of all transcripts on a per needle basis were also low in mature leaves in the second, as well as the third, year (Fig. 4B). However, the reductions in levels were not the same. The transcript of psbA was maintained at a level equal to more than 85% of that in current needles while the level of transcripts of psaA/B decreased to around 30% of that in current needles. Levels of cab, rbcS and rbcL transcripts in 2-year-old needles decreased to around 50%, 50% and 65%, respectively, of those in current needles. Together with a slight decline in levels of chloroplast proteins, the low levels of mRNAs in 2-year-old leaves suggested that senescence started in needles early in the third year. Symptoms of senescence were prominent in the PSI complex while levels of PSII and LHCII remained unaltered.

General considerations—A comparison of levels of chloroplast proteins and of mRNAs in leaves of three different ages provided a rough picture of the life history of leaves of a Japanese black pine tree. Sprouts expand with the active development of chloroplasts, with synthesis of chloroplast proteins de novo, in the first year. The development terminates within the first year and leaves reach maturity. Leaves remain in the mature state during the second year. Leaf senescence seems to begin early in the third year and progresses during the course of the third year. The picture is basically the same as that derived from previous physiological studies (Clark 1961, Kramer and Kozlowski 1979). However, the present study adds new insights that provide details of leaf life at a molecular level. Increases in levels during chloroplast development and

![Fig. 3](image-url) Northern blot of cab, psbA, psaA-psaB, rbcS and rbcL mRNAs. Equal amounts of RNA (10 μg) from current (C), 1-year-old (1) and 2-year-old (2) pine needles were loaded on a 1% agarose gel. Bands were detected by staining with ethidium bromide. They were transferred to nylon membranes and allowed to hybridize with P-labeled DNAs (pine cab cDNA, tobacco psbA and psaA-psaB, and pine rbcS cDNA and rbcL) as indicated. For details, see the text.

![Fig. 4](image-url) Changes in levels of cab, psbA, psaA-psaB, rbcS and rbcL mRNAs as needles aged. The results in Figure 3 were analyzed quantitatively, with the value for the mRNA in current needles taken as 100. Relative amounts of mRNA (A) are means ± SE of results from four different experiments. Levels of mRNA on a per needle basis (B) were calculated from data in panel A and the average abundance of RNA per needle shown in Table 1.
decreases during chloroplast senescence seem not necessarily to occur synchronously for every chloroplast component. Significant reductions in ratios of PSI/PSII and Chl a/b during chloroplast development in the first year suggest that the PSII complex and its light-harvester, LHCII, develop more slowly than the PSI complex. A significant reduction in levels of PSI and Cyt b6/f complexes without decreases in those of PSII and LHCII in 2-year-old needles also suggests that PSI and Cyt b6/f complexes decay before PSII and LHCII during the initial period of senescence.

A reduction in levels of transcripts of psaA/B in needles upon reaching maturity and entry into the senescent phase was prominent, and levels were only a little above 30% of the level in current needles (Fig. 4B). Despite such a low level of these mRNAs, PSI was maintained at a high level even in leaves during the third year. This feature suggests that the polypeptides of the PSI complex, or at least the core polypeptides, are stable in vivo. Conversely, an exceptionally high level of the mRNA for the D1 polypeptide, the psbA transcript, in mature leaves suggests the rapid turnover of the D1 polypeptide, a core polypeptide of the PSII complex (Mullet 1993). In conclusion, the expression of nuclear and chloroplast genes in pine leaves of an adult tree is related to the developmental stage of the leaves and plastids, as is also the case in pine cotyledons (Shinozaka et al. 1992a).

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References


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