Diurnal regulation of plastid genes in *Populus deltoides*

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Light regulates leaf and chloroplast development, together with overall chloroplast gene expression at various levels. Plants respond to diurnal and seasonal changes in light by changing expression of photosynthesis genes and metabolism. In *Populus deltoides*, a deciduous tree species, leaf development begins in the month of March and leaf maturation is attained by summer, which is subsequently followed by autumnal senescence and fall. In the present study, diurnal changes in the steady state transcript levels of plastid genes were examined in the fully developed leaves during summer season. Different results show that steady state level of the *psaA*/*B*, *psbA*, *psbF*/*L* and *petA* transcripts showed differential accumulation during diurnal cycle in summer. However, there was no significant change in the pigment composition during the day/night cycle.

Our studies suggest that the diurnal regulation of steady state mRNA accumulation may play a crucial role during daily adjustments in plants life with rapidly changing light irradiance and temperature.

Plants adjust to the changing irradiance and temperature during the day and different seasons by changes in different photosynthetic parameters. These adjustments reflect the changes in internal physiological factors and metabolism. These physiological processes in higher plants are characterized by rhythms, which correlate with the diurnal (light/dark) cycle. Selected biological rhythms can also be classified as circadian (related to the 24 hr cycle of earth's rotation). These processes are modulated at various levels ranging from endogenous fluctuations of plastid DNA topology, transcription, de novo synthesis of proteins to post-translational modifications of proteins.

The first evidence that the nuclear genes coding for thylakoid proteins are under the control of the "circadian clock" was provided for the *Lhcb* family encoding light-harvesting complex polypeptides. The finding that the *Lhcb* mRNA in barley fluctuates during the day has been confirmed and extended for various other plant species. In spinach, levels of mRNAs coding for proteins that belong to the same multiprotein complex generally oscillate in parallel and exhibit maxima that are specific for that complex. The mRNAs for PSI proteins appear prior to those for PSII polypeptides and these, in turn, appear prior to mRNAs for the three polypeptides constituting the oxygen evolving complex. For mRNA that change with high amplitude (e.g., LHCP) oscillations have also been found under constant conditions, indicating that a circadian oscillator is involved. Transgenic tobacco seedlings harboring chimeric GUS gene fusions with 5'-flanking sequences from the spinach genes *lhcB*, *psaF* and *atpD* confirm that the differences in the amplitude as well as the time points of maximum mRNA accumulation are perceived via cis-regulatory elements located upstream of the respective ATG codons. Minor fluctuations in mRNA levels were reported in case of *rbcS*, *rbcL*, and *psbA* and *tufA* genes in tomato leaves. Pichulla and Gruissem have documented that the expression of plastid genes, *psaA*, *psbA* and *psbB* in developing tomato fruits is partially controlled by diurnal rhythms.

*Populus deltoides* (poplar) is a perennial, deciduous tree species, which grows well in varying range of agroclimatic conditions and has been proved useful in agroforestry. In earlier studies diurnal and seasonal changes were observed in photosynthetic characteristics of poplar leaves. These changes include varying CO₂ compensation concentrations, shift in temperature optima, changes in rates of electron transport and in pigment composition. Photosystem II, is the primary target which, responds to changes in the envi-p
ronment at various levels ranging from light-induced transcription and translation of photosystem II genes to repair and degradation of specific subunits, while photosystem I remains relatively stable. In the present study, changes in steady state levels of the poplar psaA/B, psbA, psbEFLJ and petA transcripts were studied during diurnal cycle in summer. The PsA and PsB polypeptides encoded by psaA and psbA genes form the heterodimeric core of photosystem I reaction centre. The pshA gene encodes D1 protein, which together with D2 protein and cytochrome bs, forms the core of photosystem II. The psbE and psbF genes encode α and β subunits of cytochrome b599. The psbL and psbJ genes encode PsbL and PsbJ proteins respectively, also associated with photosystem II, while petA gene encodes cytochrome f, an essential component of cytochrome bo complex.

We have previously reported differential expression of psbEFLJ and petG-orf31 operons during leaf development and expression of psaA/B, psbA, psbD/C genes during autumnal senescence. Our present studies show diurnal variation in the steady state levels of psaA/B, psbA, psbEFLJ and petA transcripts.

Materials and Methods

Plant material

Populus deltoides (Stoneville, clone D121), commonly known as poplar or cottonwood tree, is a clonally propagated, shallow rooted, deciduous and fast growing tree of family Salicaceae. The present studies were carried on a two-year-old tree, growing in the field of National Botanical Research Institute, Lucknow, India.

RNA isolation and northern hybridization

In order to determine diurnal changes in steady state levels of psaA/B, psbA, psbEFLJ and petA mRNA, total RNA was isolated at different time points of the day in summer as described earlier. RNA was separated on a denaturing 1.2% agarose MOPS-formaldehyde gel and transferred to Zeta-probe membrane (Biorad). The psaA/B heterologous probe from spinach (a gift from Prof. R. G. Herrmann, Germany) and homologous gene specific probes for psbEFLJ, petA and 23S rDNA were radio-labelled and hybridized. The psbA specific 21-mer oligonucleotides obtained from Dr. Udo Johanningmeier, Germany were radio-labelled and hybridized. Same blot was used for hybridization with different probes by stripping the previous probe each time.

Estimation of photosynthetic pigments

Photosynthetic pigments namely chlorophyll and carotenoids were extracted from the leaves during different time points of the day. Chlorophyll content was estimated as described by Arnon and carotenoids were calculated according to Duxbury and Yentsch.

Results and Discussion

Our results show the response of poplar chloroplastic genes to the diurnal cycle. The steady state levels of psaA/B, psbA, psbEFLJ and petA transcripts were studied in addition to the changes in pigment composition at different time points of the day. The pigment composition of the poplar leaves remains more or less stable during the day with minor fluctuations (Fig. 1).

Diurnal profile of Photosystem gene(s) expression

In poplar psaA/B, psbA and psbEFLJ operons give rise to single transcripts of 5.8, 1.5 and 1.3 kb respectively (Fig. 2A). Previous studies demonstrated that these polycistronic messages are not subjected to further processing into smaller transcripts. The 23S rRNA probe was used to confirm equal loading of RNA in all the lanes since the synthesis of chloroplast rRNA is known to reach a steady state level at early stage in leaf development and remains more or less stable afterwards. In concurrence with these studies, the 3.4 kb 23S rRNA transcripts were present more or less in similar amounts at all the time points of the day (Fig. 2). However, the steady state tran-
Fig. 2—Diurnal fluctuation in steady state transcript levels of photosystem genes. Changes in steady state levels of psaA/B, psbA, psbEFLJ and 23S rRNA transcripts during the 24 hr day/night cycle in summer season. (A): Diurnal changes in steady state transcript levels of 23S rRNA and psbEFLJ in autumn. (B): Sizes of the transcripts are shown in kb.

script levels of psaA/B, psbA, and psbEFLJ showed diurnal fluctuations (Fig. 2A). The transcript levels of psaA/B showed a steep decline at 7 hr as compared to the 5 hr and then again showed an increase between 9 to 11 hr. The levels of psaA/B transcript did not change significantly after 11 hr except a dip at 19 hr and accumulation in night. Similarly, psbA and psbEFLJ steady state transcript levels were lower at 7 hr in comparison to 5 hr. As shown in Fig. 2A, the levels of photosystem II transcripts increase around 9 hr before a dip again between 11 to 13 hr and remains more or less stable afterwards and tends to accumulate in the night. The decrease in photosystem II transcript levels noticed at 7 hr and 11 hr were substantial and seems to be a characteristic feature of diurnal expression in summer. On the contrary, in autumn transcript level of psbEFLJ operon remains more or less constant up to 11 hr and then showed a steady accumulation (Fig. 2B). The overall levels of psbEFLJ transcript were found to be significantly higher in summer as compared to autumn (Fig. 2). Likewise, the steady state transcript levels of psbA,
psbD/C and psaA/B were reported to be low during autumnal senescence in poplar.

In general, steady state transcript levels of psaA/B, psbA and psbEFLJ exhibit maxima in the night. The diurnal fluctuation in transcripts of nuclear encoded photosynthesis genes like \( \text{Lhcb} \) and \( \text{cab} \) gene families have been widely studied\(^{11,15,16} \). However, studies related to plastid encoded genes are limited. Among plastid genes diurnal fluctuations in steady state transcript levels have been reported for \( \text{psbA} \), \( \text{psbB} \), \( \text{psaA} \) and \( \text{rbcL} \) from tomato, where low mRNA levels were consistently observed in the afternoon followed by an increased mRNA accumulation in the evening\(^{15} \). The fluctuation in chloroplastic transcript levels in poplar during the day resembles diurnal expression of tomato plastid genes documented earlier\(^{17} \) while poplar differs from tomato in the dynamics of steady state mRNA accumulation during morning hours. The major drop in photosystem I and photosystem II transcript levels at 7 hr can be correlated to light induced degradation of transcripts accumulated during night, which is then followed by \textit{de novo} synthesis of transcripts. Light induced increase in the transcription of \( \text{psbA} \), \( \text{psbEFLJ} \) and several other plastid genes have been shown, although the transcripts are present in the etioplast or in dark\(^{17} \). Seasonal variations in diurnal expression pattern of various genes could be due to either phase shift or variation in the length of the day. Alternatively, seasonal variation of the diurnal rhythms could be a result of temperature change. It has been reported that leaves primarily respond to the seasonal shift of temperature optimum for photosynthesis to deal with changing irradiance and temperature\(^4 \).

**Diurnal expression of petA gene encoding apocytochrome f**

The \textit{petA} gene probe hybridizes to ten mRNA species of 4.5, 3.6, 3.3, 3.2, 2.6, 2.5, 2.4, 1.7, 1.5 and 1.3 kb in size in poplar (Fig. 3) and show a complex transcript profile. The \textit{petA} transcribes along with \textit{psal} and \textit{ORF31} genes, giving rise to a ~ 4.5 kb polycistronic transcript. The 4.5 kb transcript is subjected to various post-transcriptional processing resulting in smaller mono and oligocistronic transcripts. The \textit{ORF231} (homologue of tobacco \textit{ORF229} and pea \textit{ORF231}) and \textit{psal} genes are located immediately upstream of \textit{petA} gene in poplar (Nathani et al. unpublished results). The pattern for \textit{petA} gene expression has been shown to be highly complex, in tobacco and pea. Out of many mRNA species, two major transcript species were detected: a 1.8 kb mRNA reflecting monocistronic \textit{petA} message and a 5.35 kb mRNA representing a polycistronic precursor transcript in tobacco\(^{27} \). The \textit{co}-transcription of \textit{petA}-\textit{ORF231} has been determined in pea, which gives rise to multiple transcripts (5.5 kb, 4.3 kb, 3.4 kb and 2.7 kb) after post-transcriptional processing\(^{28} \).

As opposed to photosystem genes, higher levels of \textit{petA} transcript were observed at 5 and 7 hr, however, the steady state levels of \textit{petA} were found to be lowest during night suggesting \textit{de novo} synthesis after sunrise. As shown in Fig. 3, the maxima in steady state levels of \textit{petA} transcripts at 7 hr was then followed by a dip at 9 hr and subsequent selective stabilization of smaller transcripts. The 2.6, 2.5, 2.4 and 1.7 kb sized transcripts of \textit{petA} showed maximum fluctuation in their levels exhibiting maxima at 19 hr (Fig. 3). It remains to be investigated whether the smaller transcripts hybridizing to \textit{petA} arise from light induced post-transcriptional processing of bigger transcripts or due to selective stabilization of smaller transcripts. The post-transcriptional processing and selective stabilization of monocistronic \textit{petA} transcripts may facilitate differential synthesis of gene

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**Fig. 3**—Changes in steady state levels of \textit{petA} transcripts during 24 hr day/night cycle. [Northern analysis was carried out using total RNA isolated from the leaves at different time points of the day in summer. Radiolabeled gene specific probes were used for hybridization].
products, encoded within the same operon during changing environmental and developmental conditions. The petA gene encodes cytochrome f subunit of cytochrome b6f complex. Interestingly, all plastid genes encoding subunits of Cytochrome b6f complex are clustered together with genes encoding components of different multisubunit complexes and exhibit complex transcript profile. Cytochrome b6f complex is composed of six subunits; cytochrome f, cytochrome b6, the Rieske-iron-sulphur protein, subunit IV, subunit V and subunit VI. The Rieske-iron-sulphur protein is encoded by nuclear genome. The genes encoding subunits cytochrome b6 and subunit IV (petB-petD) are transcribed as a part of psbB-psbT-psbH-petB-petD polycistronic message and undergo a complex post-transcriptional processing generating as many as 14 transcripts ranging from 5.5 kb to 220 nucleotides in poplar. The psbB-petD dicistronic message shows differential stabilization as compared to psbB-psbT-psbH mono and oligocistronic transcripts. The petG gene encoding subunit V of cytochrome b6f complex transcribes as ycf7-petG-ORF42 polycistrionic message in mature leaves and as a monocistronic petG transcript in developing poplar leaves. A newly identified subunit VI of the cytochrome b6f is encoded by plastid petN (ycf6) gene and co-transcribes with trnC resulting in multiple transcripts. The most abundant species includes two transcripts of ~1.25 and ~0.53 kb. Both transcript species are present at comparable levels in light-grown as well as in dark-adapted plants. The functional significance of the clustering of genes encoding subunits of different multisubunit complexes has not been understood but complex processing events generating multiple mRNA species and their selective transcription or stabilization may facilitate desired stoichiometric production of related substrates.

Our study shows variations in steady state mRNA level of photosystem I (psaA/B) and photosystem II (psbA and psbEFLJ) genes. The polycistronic psaA/B, psbA and psbEFLJ operons transcribed as single polycistronic transcript were not subjected to post-transcriptional processing leading to smaller transcript species in poplar. The petA gene encoding apocytochrome f subunit of cytochrome b6f complex shows a complex transcript profile as a result of post-transcriptional processing of polycistrionic transcript. The ratio of bigger polycistronic message and the processed mono or oligocistronic mRNA species varies during the diurnal cycle, suggesting light-induced selective accumulation of monocistronic petA transcript. The steady state mRNA levels at any given time point, represent a dynamic equilibrium between interrelated processes; such as transcription, post-transcriptional processing, RNA stability and degradation. The regulation of gene expression at various levels in changing environmental and climatic conditions is crucial for the survival of the plant. Differential accumulation of steady state transcript levels may be an important step of the complex regulatory processes to achieve required expression of photosynthesis genes.

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