Macroarray for studying chloroplast gene expression profiles associated with the initial development of wheat

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ABSTRACT

Chloroplasts, as photosynthetic carbon assimilating organelles, play an essential role in plants throughout their development. We have previously devised a mitochondrial macroarray using a set of rice and wheat mitochondrial genes to study their expression associated with biogenesis and initial development of this energy supplying organelle in wheat (1). We now report a chloroplast macroarray that can provide us with a useful means for studying chloroplast gene expression in wheat and other cereals. The chloroplast macroarray was constructed using 60 wheat chloroplast genes excluding tRNA and other small sized genes. In addition, five nuclear encoded and chloroplast-targeted genes and two control nuclear genes were included. Primer sets for the target genes were designed based on the published wheat chloroplast genome sequence (2). Changes in gene expression levels were monitored using RNA isolated from germinating seeds and seedlings at three different stages of development, i.e. at 1st, 2nd and 3rd leaf stages. Remarkable differences and dynamic changes were revealed in the amount of individual transcripts during the initial stages of wheat development. The amount of *psbA* transcript increased until the 3rd leaf stage. Those of *psaA*, *psaB* and *psaC* also increased significantly. Heterologous hybridisation was successful with cDNAs constructed from barley and rice seedlings, suggesting the versatility of this macroarray system for studies of chloroplast biogenesis and developmental changes in chloroplast gene expression in Gramineae. This system, after refining detailed protocol, can be utilized to depict chloroplast/plastid gene expression dynamics at different developmental stages and under various environmental conditions in cereals

INTRODUCTION

In the last decades molecular biology has made marked advances in understanding many important aspects of biology by providing sequence information of not only nuclear genomes but also chloroplast and mitochondrial genomes in a variety of organisms. Availability of organellar DNA sequences of many land plants has opened the perspectives for the large-scale analysis of organellar gene expression. Organellar microarray and macroarray are two powerful tools for analysing global gene expression profiles of organellar genomes at different developmental stages and under various environmental conditions in plants. In microarray, a set of gene probes corresponding to their genomic sequences, cDNA clones, or synthetic oligonucleotides each corresponding to a single specific coding sequence are spotted at a high density on a chemically coated glass slide and used as the target for different types of RNA or cDNA derived from plants grown under experimentally controlled conditions (3). In macroarray, the same kinds of probes are applied onto nylon membrane. Studies of organellar gene expression therefore have now a tendency to be built upon these two array systems, followed by northern-blot, RT-PCR or real-time PCR analysis for verification of the array data.

Recently plastid microarrays have been reported for *Arabidopsis* (4), *Chlamydomonas* (5), *Cyanidioschyzon* (6), tobacco (7), maize (8) and tomato (9). Using these microarray systems, various biological questions have been addressed such as chloroplast gene expression profiles in mutants deficient in some chloroplast transcription components in *Arabidopsis* (4). In *Chlamydomonas reinhardtii*, the specificity of a chloroplast RNA stability mutant was confirmed (5). Chloroplast gene expression profiles under dark-light changes in tobacco (7) and *Cyanidioschyzon merolae* (6) as well as plastid transcriptomes during fruit development and chloroplast to chromoplast differentiation in tomato (9) were also analysed by this system. In maize, transcript abundance of nuclear, chloroplast and mitochondrial genes was studied at different developmental stages (8).

A macroarray-based approach was also useful for comparative analysis of plastid transcriptomes in wild-type and PEP-deficient tobacco, showing that the plastid genome was completely transcribed in both types of plastids (10).

Previously we have reported the development and successful use of a mitochondrial macroarray bearing a set of rice and wheat mitochondrial genes for studying gene expression profiles associated with mitochondrial biogenesis and initial development of mitochondrial function during germination and early seedling growth in wheat (1). Now we have designed a chloroplast macroarray that can be useful in various studies of chloroplast/plastid gene expression not only in wheat but also in other cereals because it can hybridize successfully with barley and rice cDNA probes.

MATERIALS AND METHODS

The chloroplast macroarray was designed to cover 60 wheat chloroplast genes, 5 nuclear encoded chloroplast–targeted genes and 2 control nuclear genes. Primer sets

for individual genes were developed based on the published wheat chloroplast genome sequence (2) using Gene Runner3 and Olygo3 softwares. The length of amplified gene regions ranged from 200bp to 1,259bp with most probes in the size interval of 250-500bp (the sequences of all primer sets are available upon request to the authors). Amplification was carried out using total DNA and cDNA extracted from common wheat cultivars "Miranovskaya 808" and "Chinese Spring", respectively. Amplification was performed according to the following procedures: an initial denaturation step for 5 min at 94 ^{o}C followed by 25-30 cycles of denaturation for 30-50 sec at 94 ^{o}C, then annealing for 30-60 sec at 53-61 ^{o}C (primer dependent), elongation for 40-60 sec at 72 ^{o}C, with a final elongation for 5 min at 72 ^{o}C. The amplified fragments were fractionated by electrophoresis through 1.2 % agarose gel and visualized by staining with ethidium bromide for size verification. Excess primers were removed by a microSpin S-400 MR column (GE Healthcare) according to the manufacturer's protocol. After estimating concentrations of PCR products using a capillary photometer (NanoDrop Technologies), 50 ng of each dissolved in a total volume of 2.5 µl water and an equal volume of 20 x SSC was added to the nylon membrane (6.5 cm x 5 cm; Hybond^{TM}-N^{+}). PCR products were spotted 5 times manually at 1 µl each time onto the nylon membrane. The membranes were treated for denaturation of double stranded DNA according to the standard protocols.

Changes in gene expression profiles were monitored using RNA isolated from seedlings at different stages of development, i.e. embryos just after imbibition (indicated as 1 in all Figures), seedlings at 1st leaf stage on the 4th day (indicated as 2), 2nd leaf stage on the 8th day (indicated as 3) and 3rd leaf stage on the 11th day of development (shown as 4) after imbibition. Total RNA was extracted with Sepasol-RNAI and cDNA was synthesized using random hexamers and ReverTra Ace (Toyobo, Tokyo, Japan). cDNA labelling and signal detection was performed according to protocols of ECL Direct Nucleic Acid Labelling and Detection Systems (GE Healthcare). After detection step, the membrane was exposed to x-ray film (Fuji) for 3-8 h. Data was quantified by Image J software (http//rsb.info.nih.gov/ij). Mean values were normalized with respect to the actin transcript signal intensity on the same membrane. The experiment was repeated at least twice. RT-PCR was carried out in order to verify the macroarray data.

RESULTS AND DISCUSSION

After testing various experimental conditions, we applied the chloroplast macroarray to monitor changes in chloroplast transcript levels during 11 days of wheat development following imbibition. Germinating embryos and developing seedlings were incubated under 16 h light and 8 h dark conditions. Although considerable fluctuations were noted in transcript levels of individual genes, significant and consistent increases of RNA levels were detected for genes that function in photosystems I (PSI) and II (PSII) after imbibition until the 4th and/or the 8th day of development. Such up-regulated genes included *psaA*, *psaB* and *psaC* in PSI and *psbA* in PSII. On the other hand, the levels of transcripts of *ndh* and *atp* genes initially was high but then showed either no changes or decreased.

Based on the macroarray data, the level of *psbA* transcripts increased more than 30 % after imbibition till the 1st leaf stage, and then it continued to increase but more slowly to the 2nd and the 3rd leaf stages with a total increase of ca. 70 % (Fig. 1). The result obtained by the macroarray was verified by RT-PCR, although the level of increase estimated by RT-PCR was much greater (ca. 3-fold) (Figs. 2, 3). This is probably due to a lower dynamic range of the array analysis than that of PCR analysis. In *Arabidopsis*, it was reported that the level of *psbA* transcript increased two-fold from the youngest (Cot5, 2-days-old) to the oldest cotyledons (Cot30, 27-days-old) (11). Our result showing an increased level of

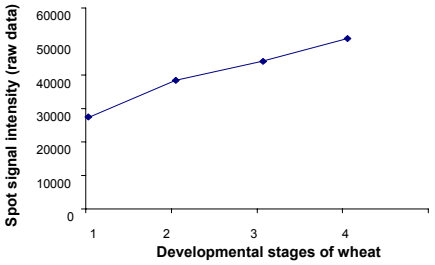

Fig. 1. Macroarray data for the transcript level of *psbA* gene during wheat development. For stages, see M & M.

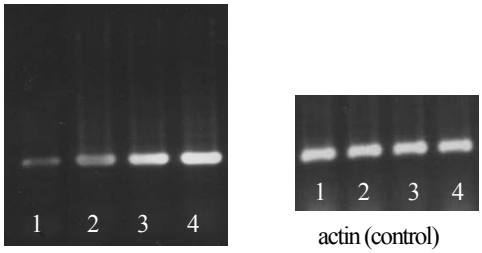

Fig. 2. RT-PCR analysis for estimating the *psbA* transcript abundance during wheat development. Images of 1. 2 % agarose gels stained with EtBr. For stages, see M & M.

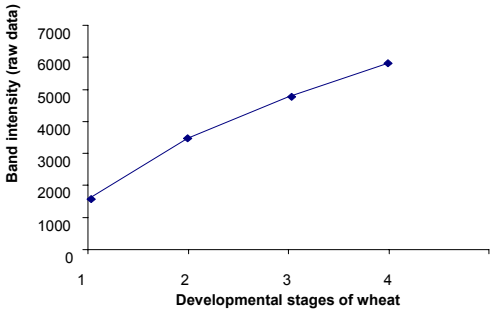

Fig. 3. Graphical view of changes in the transcript level of the *psbA* gene by RT-PCR evaluated by ImageJ software.

psbA transcript during wheat development thus agreed with the result reported in *Arabidopsis*.

The transcript levels of genes belonging to PSI at these initial stages were much lower than those of PSII genes. The highest augmentation in the expression levels (≥2-fold) was observed for *psaA*, *psaB* and *psaC* genes from imbibition till the 1st leaf stage. The rate of increase in these transcripts, however, slowed down after the 1st leaf stage until the 2nd leaf stage (Fig. 4).

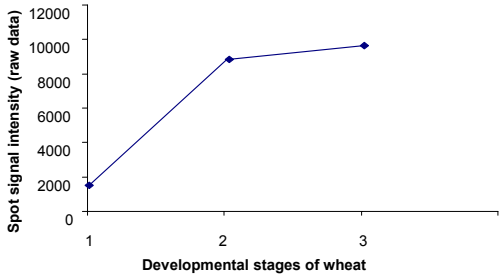

Fig. 4. Macroarray data for the transcript level of *psaA* gene during wheat development. For stages, see M & M.

To test versatility of the chloroplast macroarray system, we tried to hybridize wheat chloroplast target genes to cDNAs of barley and rice seedlings and successfully obtained clear hybridization signals with both. Fig. 5 shows an example of the hybridization signal pattern with barley cDNA. Large differences in the abundance of individual gene transcripts was apparent, indicating dynamic changes in the chloroplast gene expression profiles in cereals.

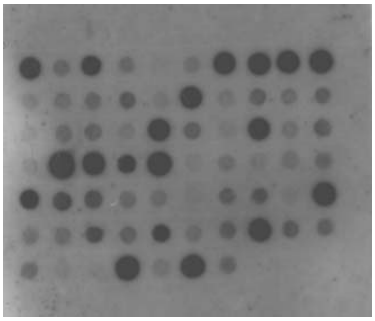

Fig. 5. Wheat plastidial macroarray showing hybridization with cDNA of barley.

CONCLUSION

Herein we reported the chloroplast macroarray system that can be applied to analyse chloroplast gene expression during imbibition to early stages of wheat development. After refining the experimental conditions such as probe spotting and signal detection procedures, this macroarray system together with the already established mitochondrial macroarray system (8), should provide useful experimental tools for studying organellar biogenesis in Gramineae.

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