BIOLOGIA PLANTARUM 56 (1): 126-130, 2012

BRIEF COMMUNICATION

Expression patterns of cotton chloroplast genes during development: implications for development of plastid transformation vectors

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Abstract

Although most plastid transformation studies have focused on chloroplast expression, plastid transformation can also be used to express genes in plastids of a wide variety of plant tissues by using appropriate plastid promoters. Based on the sequence of the *Gossypium hirsutum* chloroplast genome, we developed primers and amplified segments of 20 different plastid genes. The PCR products were labeled and used in filter dot blot hybridization studies to characterize their expression levels and patterns in total RNA isolated from light- and dark-grown cotton tissues at different developmental stages. A subset of 6 genes among these was further characterized by real time PCR. Highest expression levels were observed for *rrn16* and *psbA*. Four genes were expressed in all samples at relatively constant levels: *accD*, *atpA*, *matK* and *rrn16*. Expression in root tissue was generally low. The results of our study can be used to predict which operons and promoters are most likely to be preferentially expressed in the plastids of tissues of interest at levels that would result in the desired phenotype, facilitating the development of plastid transformation vectors.

Additional key words: chloroplast, *Gossypium hirsutum,* PCR, promoters, transcriptional regulation.

Expression of nuclear-encoded transgenes in plants can be limited by gene silencing and position effects. Some gene products are toxic when expressed at high levels in the plant cell cytoplasm and the potential for horizontal transfer of transgenes by pollen transmission is an inherent environmental hazard (Daniell *et al.* 2005). Plastid engineering circumvents many of these concerns. High levels of transgene expression are possible in transplastomic plants due to the presence of multiple plastids within cells, each containing multiple copies of the genome and because gene silencing does not occur in plastids (Daniell 1993, DeCosa *et al.* 2001). Other advantages associated with plastid transformation include efficient expression of bacterial genes, expression of suites of genes from a single promoter (operon; Bock 2007), elimination of excess flanking sequences and marker genes (Daniell *et al.* 2001), and containment of transgenes.

We are interested in developing cotton with enhanced resistance to fungal pathogens including *Aspergillus flavus*. Although we have identified structural genes whose products reduce *A. flavus* growth (Rajasekaran *et al.* 2005, 2008), higher resistance are needed for effective control of fungal infection in the field. Since higher expression levels are associated with higher resistance, we are interested in developing cotton plastid transformation vectors to increase the levels of antifungal gene expression in plastids in a variety of tissues. Chloroplast transformation has become routine for tobacco (Svab and Maliga 1993, Daniell *et al.* 2005) and has been reported for a number of other plants including carrot (Kumar *et al.* 2004a), soybean (Dufourmantel *et al.* 2004, 2006), rice (Lee *et al.* 2006), potato (Sidorov *et al.* 1999), tomato (Ruf *et al.* 2001) and cotton (Kumar *et al.* 2004b).

The chloroplast ribosomal RNA promoter has

Received 19 July 2010, *accepted* 8 December 2010.
Abbreviations: CD, CL - dark and light-grown cotyledon; DS - mature cotton seed; dpa - days post anthesis; ER - cotyledons from the emerging radicle stage; HD, HL - dark and light-grown hypocotyl; L - mature leaf; PCR - polymerase chain reacion; PS - photosystem; RD, RL - dark and light-grown root.
Acknowledgements: USDA/ARS Cooperative Research Agreement 58-64-35-8-300, 2009-2011.
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commonly been used to obtain high level, constitutive transgene expression in chloroplasts of transplastomic plants (McBride *et al.* 1995, Daniell *et al.* 1998, Kota *et al.* 1999, Iamtham and Day 2000, De Cosa *et al.* 2001, Ruiz *et al.* 2003). However, we need to express antifungal genes in both green and non-green tissues throughout vegetative growth and seed development. To identify promoters and operons that meet these criteria, we have characterized the RNA expression patterns of 20 plastid genes. Total RNA extracted from cotyledons of developing cotton embryos (28, 38 and 48 d post anthesis; dpa), mature cotton seed (DS), cotyledons from the emerging radicle stage (ER), mature leaf (L) and light and dark-grown tissues of hypocotyl (HL, HD), root (RL, RD) and cotyledon (CL, CD) was screened for expression of these genes by hybridization analyses. We chose 6 of those genes for quantitative analyses to determine expression levels of these genes relative to that of an RNA polymerase gene, *rpoB*. The results of these studies and their potential applications are presented.

Cotton bolls were harvested from greenhouse-grown *Gossypium hirsutum* L. cv. Coker 312 plants at 28, 38 and 48 dpa. Immature cotyledons were removed and immediately frozen in liquid nitrogen. Dry seed was collected from mature bolls and mature leaf tissue was collected from greenhouse-grown plants. Seeds were surface-sterilized, transferred to agar-solidified Stewart's medium (Stewart and Hsu 1977) without sucrose, pH 6.8, in 2.5 × 20 cm tubes and incubated in a growth chamber at 30 °C with a 16-h photoperiod (irradiance of 250 μmol m^{-2} s^{-1}). As soon as the radicle emerged, the seed coat was removed and the cotyledons from this stage were harvested and quick frozen in liquid nitrogen. After 7 to 10 d of incubation, cotyledon, hypocotyl and root tissues were harvested from the light- and dark-grown seedlings and frozen immediately in liquid nitrogen.

Total RNA was prepared from cotyledons of developing embryos and dry seeds as described (Galau *et al.* 1981). Total RNA was extracted from all other vegetative tissues using the *Spectrum* plant total RNA extraction kit (*Sigma*, St. Louis, USA). RNA was quantified using a *Nanodrop 1000* spectrophotometer (*Nanodrop Technologies Inc*., Wilmington, USA). RNA integrity was checked by electrophoresis on denaturing agarose gels.

Primers were designed to amplify 20 different chloroplast gene sequences based on the published sequence of the cotton chloroplast genome (Lee *et al.* 2006) (*DNAMAN*, *Lynnon Corp*., Quebec, Canada). Cotton chloroplast sequences were amplified as follows: *Platinum PCR Supermix* 25 mm^3 (*Invitrogen*, Carlsbad, USA), 30 ng cotton chloroplast DNA (Triboush *et al.* 1998), 1 mm^3 40 mM forward and 1 mm^3 reverse 40 mM reverse primer (*Sigma*, St. Louis, USA) for 35 cycles using the manufacturer's recommended conditions (*Invitrogen*). PCR products were cloned into the vector PCR 2.1 as described (*Invitrogen*). DIG-labeled probes were generated by direct DIG-PCR labeling (*Roche Diagnostics*, Indianapolis, USA) of the following plasmid clone templates: pGhRrn16, pGhAtpB, pGhYcf1, pGhCemA, pGhRps4, pGhMatK, pGhClpP, pGhAtpA, pGhAccD, pGhPsaB, pGhNdhD, pGhYcf2, pGhRps7. When direct DIG- PCR labeling was unsuccessful (pGhYcf3, pGhYcf4, pGhRpoB, pGhRpoA, pGhRbcL, pGhPsbA and pGhNdhF), PCR was used to amplify the chloroplast sequences (*Platinum PCR Supermix*) and the PCR products were labeled with DIG-UTP using a random primed reaction (*Roche Diagnostics,* Indianapolis, USA).

Total RNA (5 μg per well) was applied to *Magnagraph* membranes (*MSI*, Westborough, USA) using a vacuum manifold (*BioRad*, Hercules, USA) as per the manufacturer's recommendations. RNA was fixed to the membranes by heating at 60 °C for 2 h. Filters were pre-hybridized in *DIG-Easy Hyb* (*Roche Diagnostics*) at 50 °C shaking ≧ 2 h and then transferred to individual bags for hybridization. After overnight hybridization at 50 °C shaking, filters were washed as described (*Genius Users Manual*, *Roche Diagnostics*), transferred to acetate sheets and treated with *CDP-Star* (*Roche Diagnostics*). Signals were detected with a *ChemiDoc XRS* (*BioRad*).

cDNA was synthesized using the *iScript* cDNA synthesis kit (*BioRad*) from 1 μg total RNA as per manufacturer's instructions. The cDNA was quantified using a *Nano Drop 1000* spectrophotometer. Primers were designed for real time studies using *Beacon Designer 3.01* (*Premier Biosoft*, Palo Alto, USA). Annealing temperatures were empirically optimized for amplification of chloroplast products with an *Icycler* and *iQ SYBR* green supermix (*BioRad*). Real time quantification was performed in 96-well plates using the following conditions: 500 ng cDNA template, 280 nM each primer and 10 mm^3 of 2× *iQ SYBR* green supermix in a final volume of 20 mm^3. The reactions were incubated for 3 min at 95 °C followed by 50 cycles of at 95 °C, 30 s at 52.1 °C, 30 s at 72 °C followed by 1 min at 52.1 °C. Melt curves were performed by increasing the temperature from 52.1 °C to 92.1 °C in increments of 0.5 °C every 10 s using an *Icycler* (*BioRad*). The calculated threshold (Ct) was determined using the maximum curvature approach. The per-well baseline cycles were automatically determined and the data analysis window was set at 95 % of a cycle, centered at the end of the cycle except for one repetition of the *rrn16* sequences in the dark grown cotyledon tissue (a user defined limit was implemented for that one sample). Weighted mean digital filtering was applied to both the data and melt curve analyses.

Three independent cDNA synthesis reactions were tested for all primer pairs and all amplifications were performed in duplicate. The Ct values for duplicate wells were averaged, and the average value was converted to number of molecules ($1/2^{Ct}$). This number was divided by

the number of molecules obtained for *rpoB* with the same cDNA sample. Comparative values for the three cDNA preparations were averaged, and the standard error calculated (*Microsoft Excel*, *Microsoft*, Redmond, USA).

Genes whose transcripts were detected at relatively consistent levels in all tissue samples included *accD, atpA*, *matK* and *rrn16* (Fig. 1). These gene products are all involved in processes that are essential for cell growth and metabolism: *accD* encodes the β-subunit of plastid acetyl CoA carboxylase, *atpA* encodes the α-subunit of the H^+ATP synthase CF1, *matK* encodes a maturase that is presumed to be involved in splicing of group II introns and *rrn16* encodes the 16S ribosomal RNA.

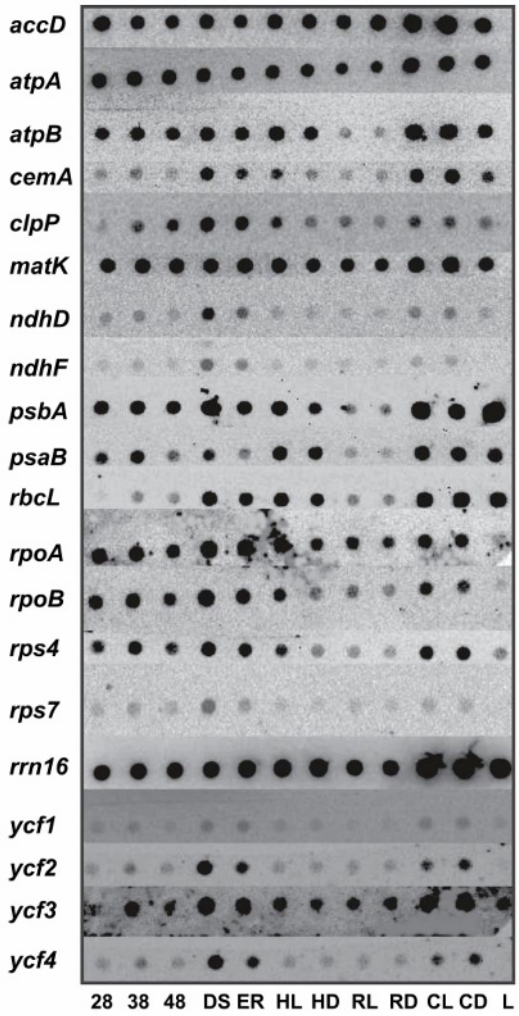

Fig. 1. Hybridization of cotton stage specific total RNA with chloroplast gene probes. Total RNA was extracted from developing embryos at 28, 38 and 48 d post anthesis (28, 38, 48), dry seeds (DS), cotyledons from the emerging radicle stage (ER), light-grown hypocotyl (HL), dark-grown hypocotyl (HD), light-grown roots (RL), dark-grown roots (RD), light-grown cotyledons (CL), dark-grown cotyledons (CD) and mature leaves (L) and applied to membranes. The membranes were hybridized with plastid probes for the genes listed on the left side of the figure.

Genes associated with photosynthetic complexes had different hybridization patterns as compared to genes associated with assembly of the complexes (Fig. 1). *psaB,* which encodes one of the major photosystem 1 (PS 1) proteins, was expressed at the highest levels in 38 dpa cotyledons, light- and dark-grown hypocotyls and cotyledons and mature leaves. The highest expression levels detected for two genes associated with assembly or stability of PS 1, *ycf3* and *ycf4*, were observed in DS, ER, CL and CD stages. Expression levels of *psbA* which encodes a PS 2 protein were high in all tissues and treatments tested except for light- and dark-grown roots. Transcription levels of the large subunit of Rubisco (*rbcL*) were most abundant in the DS, ER, HL, HD, CL, CD and L tissues.

Transcripts of *clpP* which encodes an ATP dependent subunit of the Clp protease increased in cotyledons during development and reached maximum levels at the DS and ER stages. Similar patterns of hybridization were observed for *ndhD*, *ndhF* (NADH dehydrogenase genes), *rps7* (ribosomal protein genes) and *ycf1* and *ycf2* (function unknown) and *ycf4* (thylakoid protein possibly associated with PS 1 scaffold assembly). Expression levels of *cemA* which encodes a chloroplast envelope protein were highest at the DS and mature CL and CD (Fig. 1).

Three types of tissues from plants grown in light and dark conditions were tested for the presence of specific chloroplast RNAs. Hypocotyl and cotyledon tissue total RNAs from light-grown tissue hybridize more strongly with the *psbA* probe as opposed to dark-grown tissue. Expression levels of plastid genes in CL were similar to those observed for L in nine cases (*atpA, atpB, rrn16, accD, matK, clpP, rbcL, psbA and psaB*). In all the other cases, expression was higher in mature cotyledon than in mature leaves (Fig. 1).

Total RNA from various developmental stages differentially hybridized with each of the plastid gene probes. RNA extracted from mature seeds preferentially hybridized with a number of probes including those for *cemA*, *clpP*, *ndhD*, *ndhF, rbcL, Ycf2* and *Ycf4.* (Fig. 1). Total RNA from hypocotyls hybridized with most of the probes, but probes from the light-grown tissue did not hybridize as strongly with *ndhD*, *ndhF*, *rps7, ycf1, ycf2* or *ycf4.* Expression of the plastid genes was minimal in root tissues: only *matK, accD* and *rrn16* hybridized at levels that were similar to those observed with those probes for the other tissue samples.

Expression levels of six genes (*accD*, *atpA*, *matK*, *rps7*, p*sbA* and *rrn16*) were further characterized with real time PCR. Expression levels were normalized to the levels observed for *rpoB* (Table 1). Highest expression levels in all tissues were observed for *rrn16*. Its expression level ranges from 61-fold (RL) up to 938-fold (L), of that seen for *rpoB* in the same samples. The only other gene exhibiting normalized expression levels on the same scale is *psbA*: 39 times higher than that of *rpoB* in

Table 1. Relative expression levels of selected chloroplast genes compared to expression levels of chloroplast *rpoB*. Stage specific/treatment specific cDNAs were generated from RNA preparations as described in Fig. 1. Means and standard errors shown were generated from three experiments. The mean values from each test plastid gene were normalized to the mean values obtained for *RpoB* with the same templates.

Gene	28	38	48	DS	ER	HL	HD	RL	RD	CL	CD	L
accD	0.91±0.09	0.50±0.03	0.30±0.05	0.56±0.10	0.38±0.05	1.58±0.12	2.19±0.54	2.25±0.39	2.40±0.22	2.17±0.09	1.99±0.15	1.64±0.30
atpA	1.18±0.46	1.15±0.28	0.59±0.13	0.62±0.02	0.45±0.03	1.15±0.39	1.54±0.20	1.00±0.28	1.01±0.19	2.19±0.75	1.98±0.68	4.08±1.59
matK	1.58±0.32	1.22±0.16	0.78±0.05	1.76±0.32	1.54±0.24	2.19±0.42	2.69±0.21	1.38±0.11	1.86±0.30	3.16±0.48	2.91±0.24	2.51±0.50
psbA	9.04±3.28	7.25±2.19	5.40±2.11	1.86±0.66	1.47±0.32	39.3±13.9	13.6±6.44	4.05±1.12	1.10±0.16	111 ±42.0	58.1±22.0	612 ±127
rps7	1.58±0.32	1.22±0.16	0.78±0.05	1.76±0.32	1.54±0.24	2.19±0.42	2.69±0.21	1.38±0.11	1.86±0.30	3.16±0.48	2.91±0.24	2.51±0.50
rrn16	103 ±37.8	66.9±11.5	66.9±13.9	70.1±10.8	87.4±8.22	473 ±86.2	372 ±110	61.3±12.1	64.5±14.6	703 ±191	461 ±148	938 ±318

HL, 111 times higher in CL and 612 times higher in L. The other four genes tested: *accD*, *atpA*, *matK* and *rps7* exhibited consistent, low expression levels in all tissues tested (normalized values ≤ 4× that of *rpoB*).

In this study we have identified specific genes appropriate for the development of cotton plastid transformation vectors. The highest levels of transgene expression in cotton plastids would be associated with expression from the *rrn16* operon. This promoter has been used to achieve high levels of transgene expression in many transplastomic studies (McBride *et al.* 1995, Daniell *et al.* 1998, Kota *et al.* l 1999, Iamtham and Day 2000, DeCosa *et al.* 2001, Ruiz *et al.* 2003). However, since *rrn16* is expressed at high levels in plastids of all tissues, that may not always be the best choice. For preferential expression in seed plastids, *cemA* may be a more appropriate choice: maximum expression of *cemA* was observed in dry seed and cotyledon tissues. For expression in root plastids, *accD*, *matK* and *rrn16* would be good candidates. If root expression is not desired, the *psbA* promoter would be appropriate. Low levels of constitutive expression in a wide variety of tissues could be achieved through development of vectors that use the *accD*, *atpA*, *matK* or *ycf3* promoter.

Although this study specifically assessed plastid transcript levels and patterns of plastid expression in cotton, these results can also be applied to the development of plastid transformation systems in other plant species. The expression patterns we observed can be used to predict which operons and promoters are most likely to drive preferential expression in the tissues of interest at levels resulting in the desired phenotype.

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