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A molecular approach to combat spatio-temporal variation in insecticidal gene (*Cry1Ac*) expression in cotton

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Abstract Spatio-temporal expression of an insecticidal gene (*Cry1Ac*) in pre existing transgenic lines of transgenic cotton was studied. Seasonal decline in expression of *Cry1Ac* differed significantly among different cotton lines tested in the field conditions. The leaves of the *Bt* cotton plants were found to have the highest levels of toxin expression followed by squares, bolls, anthers and petals. Expression of the gene decreased consistently with the age of plants. Toxin expression in fruiting parts was not enough to confer full resistance against bollworms. The reduction in efficacy of transgenic cotton plants late in the season was attributed to reduction in promoter activity. For this purpose, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit (*rbcS*) promoter was isolated from *Gossypium arboreum* that was further cloned upstream of an insecticidal gene (*Cry1Ac*) in expression vector pCAMBIA 1301. A local cotton cultivar NIAB-846 was transformed with *Cry1Ac* driven by *rbcS* promoter. The same cotton cultivar was also transformed with *Cry1Ac* gene driven by 35SCaMV promoter to compare the expression pattern of insecticidal gene under two different promoters. The results showed that *rbcS* is an efficient promoter to drive the expression of *Cry1Ac* gene consistent throughout the life of cotton plant as compared to 35S promoter. The use of tissue specific promoter is also useful for addressing the biosafety issues as the promoter activity is limited to green parts of plants, hence no gene expression in roots, cotton seed and other cotton products and by products.

Keywords *rbcS* Promoter · Consistent · Insecticidal gene expression · Cotton transformation

Abbreviations

CEMB	Centre of Excellence in Molecular Biology
rbcS	Rubisco small subunit
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
ELISA	Enzyme linked immunosorbent assay
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Introduction

Cotton is an important economic and fibre crop worldwide and likewise it serves as a jugular vein in the economy of Pakistan because of its major share in GDP. Transgenic cotton expressing *Bt* (*Bacillus thuringiensis*) toxins is currently cultivated on a large commercial scale in many countries, but data has

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shown that it behaves variably in toxin efficacy against target insects under field and greenhouse conditions (Benedict et al. 1996; Finnegan et al. 1998; Chen et al. 2000; Guo et al. 2001; Mahon et al. 2002; Olsen et al. 2005; Adamczyk et al. 2009; Manjunatha et al. 2009; Bakhsh et al. 2010). Understanding of the temporal and spatial variation in efficacy and the resulting mechanisms is essential for cotton protection and production.

The mechanisms of variation in endotoxin protein content in plant tissues are rather complicated. The reduction in Bt protein contents in late-season cotton tissues could be attributed to the overexpression of *Bt* gene at earlier stages, which leads to gene regulation at post-transcription levels and consequently results in gene silencing at a later stage (Xia et al. 2005). Methylation of the promotor may also play a role in the declined expression of endotoxin proteins (Xia et al. 2005; Dong and Li 2007). Several other factors might also be responsible for variation in the gene expression level, e.g. change in the nucleotide sequence of the gene, type of promoter, and the insertion point of the insert/cassette in the DNA of the transgenic variety, transgene copy number, internal cell environment, as well as several external factors in the environment (Hobbs et al. 1993; Guo et al. 2001; Rao 2009).

Nearly all transgenic crops around the world utilize the CaMV 35S promoter (or similar promoters from closely-related viruses) to drive transgenes (Odell et al. 1985). It is only now becoming clear that this promoter is not as robust as laboratory and glasshouse studies have suggested. Its function is influenced by as yet undefined physiological and perhaps environmental factors (Sunilkumar et al. 2002; Bakhsh et al. 2010). Compared with the temporal or spatial specific expression of the toxin, constitutive expression of foreign proteins in transgenic plants may cause adverse effects, such as the metabolic burden imposed on plants for constant synthesis of foreign gene products, and these may increase the potential risk of resistance of the target insects to Bt. There is also concern about the food safety of genetically modified plants (Kuiper et al. 2001; Shelton et al. 2002; Conner et al. 2003).

Consistent and targeted expression has become particularly important for the future development of value-added crops because the public may be more likely to accept 'less intrusive' expression of the transgenes. As sophistication in biotechnology improves, the need for more developmentally or environmentally regulated promoters is becoming evident and considerable efforts should be exerted for the discovery of specific tissue or biotic, hormonal or abiotic stress responsive genes and promoters (Potenza et al. 2003).

The present study was conducted to evaluate the expression of insecticidal gene (*Cry1Ac*) with the age of plants as well in different plant parts because the sustainable expression of Bt genes is crucial for its effectiveness to control the targeted insect pests. A strategy was devised out to make expression of *Cry1Ac* consistent in cotton by isolating a ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit (*rbcS*) promoter from *Gossypium arboreum* (cv. FDH-786) and was further transformed in *Gossypium hirsutum* (cv. NIAB-846).

Materials and methods

Twelve pre-existing transgenic cotton lines (cv. CIM-482) transformed with an insecticidal gene (*Cry1Ac)* (Rashid et al. 2008) were planted at Centre of Excellence in Molecular Biology (CEMB) campus experimental field along with non transgenic control using randomized complete block design with three replications.

Temporal and spatial expression of *Cry1Ac* endotoxin

As differential expression of *Cry1Ac* occurs among different plant structures (Greenplate 1999; Adamczyk et al. 2001), a single structure was selected for quantification of *Cry1Ac*. For each sample date and for all transgenic lines, a single main-stem terminal leaf was collected from five plants/transgenic line after every 15 days interval. For spatial expression, various plant parts i.e. leaf, anthers, pollens, square buds and bolls were selected at reproductive growth stage. The samples were transported to the laboratory and were processed the same day.

Expression of *Cry1Ac* in transgenic lines was quantified by enzyme linked immunosorbent assay (ELISA) using Envirologix Kit (Cat # AP051). Negative and positive controls were added to the wells along with test samples. ELISA was performed

according to procedure given in the kit and quantification of *Cry1Ac* endotoxins was done by plotting absorbance values of *Cry1Ac* test samples on the standard curve generated with purified *Cry1Ac* standards on each of ELISA plates and expressed as nanogram of *Cry1Ac* per gram of fresh tissue weight. Protein expression data of *Cry1Ac* was further subjected to statistical analysis using SPSS software (version 11.0, SPSS Inc) to evaluate the differences among transgenic lines, sampling dates, interaction of transgenic lines and sampling dates at 5% level of significance.

Leaf biotoxicity assay

Based on spatio-temporal results, leaf biotoxicity assays were performed to counter check the variation in efficacy of endotoxin *Cry1Ac* against targeted insect pests after 30, 60 and 90 days of crop age. The five leaves from the upper, middle and the lower portion of each transgenic line along with the leaves from non transgenic control line in triplicates were detached on moist filter papers in petri plates and 2nd instar *Heliothis* larvae were fed to them. After 2–3 days, mortality rates of *Heliothis* larvae were recorded. The mortality rates were calculated as follows:

$$\%\text{Mortality} = \frac{\text{No. of dead larvae}}{\text{Total no. of larvae}} \times 100$$

Isolation and sequencing of *rbcS* promoter

Total RNA extraction from leaves of cotton (*G. arboreum* cv. FDH-786) was followed using method of Jaakola et al. (2001) with some modifications. cDNA was synthesized by using Fermentas cDNA synthesis kit. The promoter region was amplified by polymerase chain reaction (PCR) using oligonucleotide primer including an KpnI restriction site in the forward and reverse primers (F: 5′-GCGGTACCCAAAAGAGTCTCACTGATCC-3′)and(R: 5′-GCGGTACCCTCTTTGTGGTCATAATGGT-3′). The promoter fragment was amplified by high fidelity PCR (1 reaction: 2.5 µl 10× buffer, 2.5 µl 2 mM dNTP, 1 µl each primer (10 µM), 1 µl *Taq* DNA polymerase, 0.5 µl DNA (100 ng/µl), 16.5 µl H_2O) under the following conditions: initial denaturation 94°C for 5 min; followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 10 min. The *rbcS* promoter region (445 bp) was confirmed by sequencing.

Plasmid construction

Plasmid construction was carried out in two steps. The *Cry1Ac* gene and NOS terminator fragment (2.11 kb) was amplified from its source plasmid PIA-2 (already available plasmid in laboratory having *Cry1Ac* under ubiquitin promoter) by PCR using BamHI and KpnI restriction sites and cloned in pre-digested plasmid pCAMBIA 1301. The binary vector pCAMBIA-1301 (11,837 bp) was used for cloning of *Cry1Ac* gene under *rbcS* promoter. The vector contains kanamycin gene for bacterial selection and hygromycin for plant selection resistance genes.

Furthermore, *rbcS* promoter was subcloned upstream of *Cry1Ac* + NOS fragment into the plasmid pCAMBIA 1301 pre-digested with KpnI. PCR, Restriction digestion and sequencing techniques were employed to confirm cloning of insert (promoter and insecticidal gene) in plant expression vector. The resulting plasmid was named *Rb-Ac* and was used to transform *Agrobacterium tumefaciens* strain LBA4404.

Cotton transformation

Cotton (*G. hirsutum*) cv. NIAB-846 was selected for transformation because it had high yield potential and desired fibre characteristics. The seeds were delinted and surface sterilized with Tween-20 for 3 min and further subjected to 0.1% $HgCl_2$ and 0.1% Sodium dodecyl sulphate (SDS) solution mixture. The sterilized seeds were placed in dark at 30°C overnight for the germination. The shoot apices of germinating seedlings were used for *Agrobacterium*-mediated transformation according to the procedure described by Gould and Magallanes-Cedeno (1998) with some modifications adopted at CEMB as described by Rao (2009), Bakhsh (2010) and Khan et al. (2011). The inoculated explants were cultured on MS medium (Murashige and Skoog 1962) containing 1 mg/l Kinetin for 3 days at 28 ± 2°C. After co-cultivation, plantlets were sub-cultured on selection medium i.e. MS containing 75 µg/ml hygromycin, also supplemented with 0.5 mg/l benzylaminopurine (BAP) and 1 mg/l α-naphthaleneacetic acid (NAA). Cefataxime (250 mg/l) was also added to inhibit bacterial overgrowth. Sub culturing was done after every 10 days.

After 2 months selection, these plantlets were shifted to hygromycin free MS medium until fully plants were developed. The putative transgenic plants were further shifted to pots containing soil of equal proportion of clay, sand and peat moss (1:1:1). Finally the plants were shifted to greenhouse and were subjected to various molecular analysis. Same cotton cultivar was also transformed with another plasmid *pk2Ac* (having *Cry1Ac* under 35S promoter) to study comparative spatio temporal expression of *Cry1Ac* in newly transformed cotton plants.

Molecular analysis of transgenic plants

Genomic DNA was isolated from newly transformed *Rb-Ac* plants (transformed with *Cry1Ac* under *rbc*S promoter) as well as *pk2Ac* plants (transformed with *Cry1Ac* under 35S promoter). PCR was run for the detection of integrated *Cry1Ac* to amplify internal fragments of 565 bp using gene specific primers by a modification of the method by Saiki et al. (1988). DNA extracted from untransformed plants was used as negative control and that of plasmid *Rb-Ac* as positive control. The PCR was performed at 94°C for 4 min, 94°C for 1 min, 52°C for 1 min and 72°C for 1 min followed by 35 cycles.

SDS-PAGE (polyacrylamide gel electrophoresis) of transgenic PCR positive *Rb-Ac* and *pk2Ac* plants was carried out by the method of Laemmli (1970) to confirm *Cry1Ac* expression. About 50 µg of isolated protein was mixed with loading dye denatured by heat shock in boiling water bath for 5 min and quick chilled on ice. Prepared 12% resolving, and 4% stacking gel in gel assembly. Stained the gel with commassie stain for 30 min and destained it with destaining solution.

Temporal and spatial expression of *Cry1Ac* in newly transformed plants

Newly transformed *Rb-Ac* and *pk2Ac* plants were further investigated to evaluate the temporal and spatial expression of *Cry1Ac* endotoxins. For each sample date, a single main-stem terminal leaf was collected from five plants/line at different intervals of plant growth stages. ELISA assay was performed using Envirologix Kit (Cat # AP051) and concentration of *Cry1Ac* gene in *Rb-Ac* and *pk2Ac* plants was quantified and expressed as nanogram per gram of fresh tissue weight. Different plants parts at reproductive stage were undertaken for spatial expression.

Results

Temporal and spatial expression of *Cry1Ac* endotoxin

Temporal expression of *Cry1Ac* gene was quantified in transgenic lines after 15 days interval. The result showed that the toxin level declined with the age of plant (Fig. 1a) being maximum at vegetative stage, 30 days crop. As the crop progressed towards the maturity, a decline in expression level of *Cry1Ac* was observed. Similar expression pattern was found in all these transgenic lines. Statistical analysis revealed that *Cry1Ac* differences among transgenic lines, sampling dates and their interaction were significant at 5% level of significance.

To evaluate spatial expression, different plant parts i.e. leaves, square buds, bolls, anthers, petals and ovary were sampled from the field and subjected to ELISA assay. The result revealed that the expression of *Cry1Ac* was variable in different plant parts, being maximum in the leaves followed by square buds, bolls and anthers (Fig. 1b). Petals were showing less toxin expression as compared to other plant parts being only 8–10 ng/g of fresh tissues weight while *Cry1Ac* expression in ovary remained undetectable.

Leaf biotoxicity assay

Laboratory biotoxicity assays with 2nd Instar *Heliothis* larvae were conducted to confirm the variation in expression of the insecticidal gene at 30, 60 and 90 days of crop age. The results showed that transgenic lines showed a varying mortality rate of *Heliothis* larvae that ranged between 60 and 90% at different growth stages. The varying mortality rates indicated a variation in *Cry1Ac* protein levels. Transgenic lines that showed 100 mortality rate of *Heliothis* larvae at 30 days crop age, showed varying 60–80% mortality rate at 90 days of crop age (Fig. 2) while in case of non transformed control CIM-482, no any larval mortality was recorded.

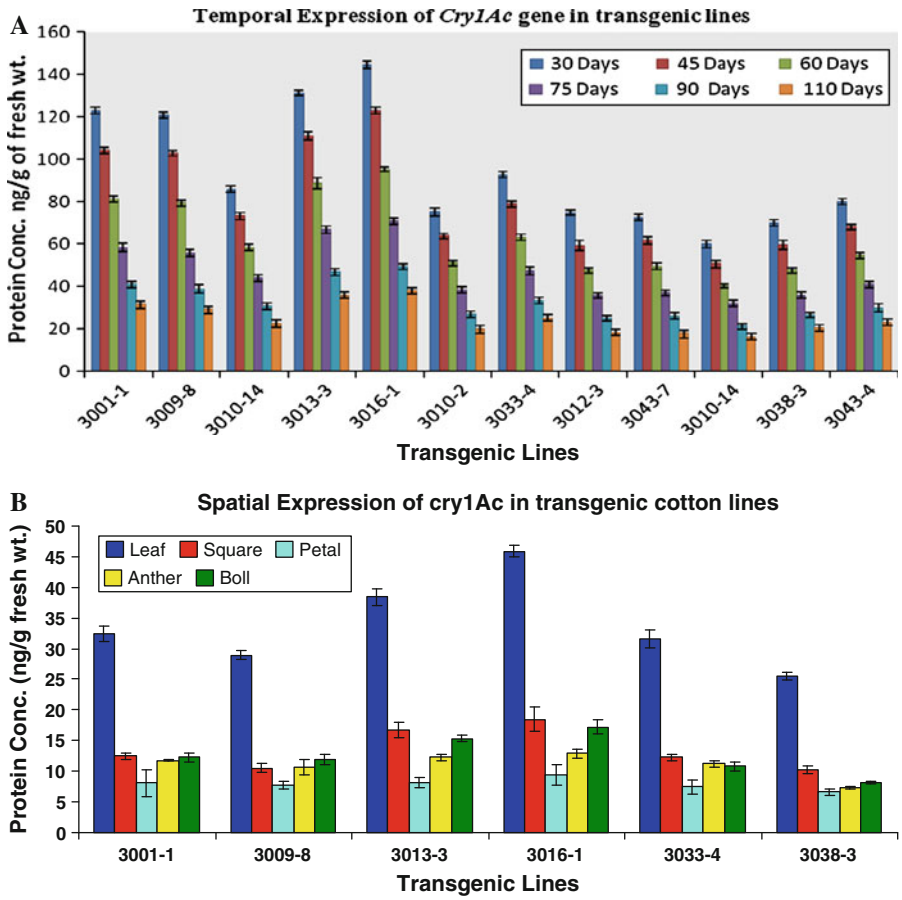

Fig. 1 Spatio temporal expression of *Cry1Ac* in transgenic cotton lines. **a** Temporal expression of *Cry1Ac* was quantified after an interval of 15 days showing the maximum expression at vegetative growth stage while a decline in expression at the later stages of crop plants was observed. **b** Spatial expression of *Cry1Ac* quantified in different plant parts showing maximum in leaf followed by followed by square buds, bolls and anthers

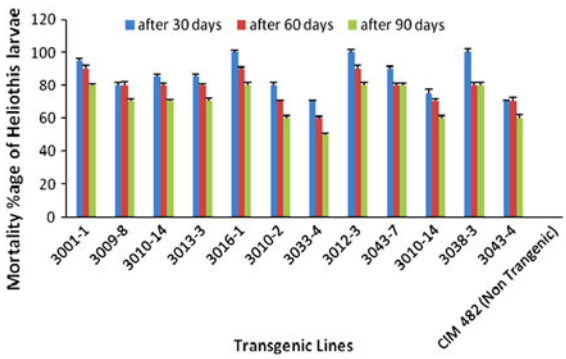

Fig. 2 Mortality % age of 2nd *Heliothis* larvae in different transgenic lines at 30, 60 and 90 days of crop plants. *Lines* showed 100% mortality of larvae at 30 days crop stage while this percentage varied at 90 days observation while no any larval mortality was recorded in non transgenic control cotton cultivar CIM-482

Cotton transformation

An optimized procedure by Gould and Magallanes-Cedeno (1998) was followed to transform cotton cultivar with some modifications (Fig. 3). A total of 4,000 mature embryos were used in all the transformation experiments. After 8 weeks of selection on 75 µg/ml hygromycin, 50 plants of *Rb-Ac* and 43 plants for *pk2Ac* plants were obtained. Among these 50 plants of *Rb-Ac* plants, 12 plants were shifted in the field while 10 *pk2Ac* plants established properly in soil. The overall transformation efficiency in both types of transgenic plants remained 0.55% (Table 1).

Confirmation of gene integration and expression

Newly transformants *Rb-Ac* and *pk2Ac* were analyzed for the integration of *Cry1Ac* gene using gene specific primer by PCR assay. The amplified fragment of 565 bp revealed that *Cry1Ac* gene was integrated in *Rb-Ac* and *pk2Ac* plants successfully while no amplification was observed in non transformed plant sample (Fig. 4a). All the transgenic plants were phenotypically normal and grew well. Expression of *Cry1Ac* protein in transgenic plants of *Rb-Ac* and

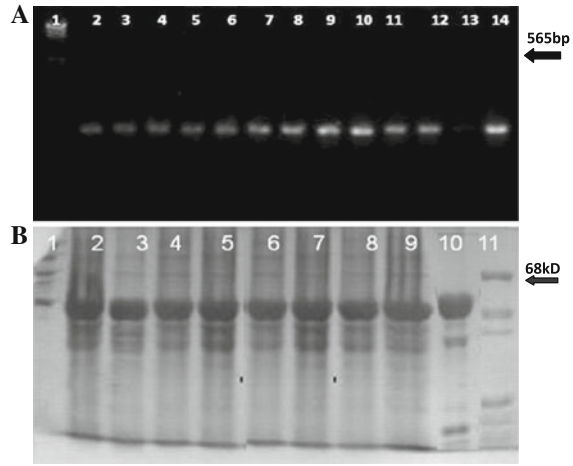

Fig. 3 Confirmation of gene integration and expression in transgenic plants. **a** PCR assay showed the amplification of required *Cry1Ac* band. *Lane 1* Lamba *Hin*dIII marker, *lane 2–7* Transgenic plants of *Rb-Ac*, *lane 8–12* transgenic plants of *pk2Ac*, *lane 13* negative control (nontransformed NIAB-846), *lane 14* positive control (plasmid DNA). **b** SDS-PAGE analysis of *Rb-Ac* and *pk2Ac* plants confirmed expression of 68 kDa band. *Lane 1* high mol. weigh protein marker (Fermentas), *lane 2–5* transgenic plants of *Rb-Ac*, *lane 6–9* transgenic plants of *pk2Ac*, *lane 10* negative control NIAB-846, *lane 11* positive control protein isolated from HD-73 strain

pk2Ac was confirmed by SDS-PAGE. A 68 kDa band of *Cry1Ac* protein was observed in all the transgenic plant samples including positive control while no such band was present in negative control (Fig. 4b); thereby confirming the expression of introduced gene (*Cry1Ac*) in transgenic plants.

Temporal and spatial expression of *Cry1Ac* in newly transformed plants

The transgenic plants of *Rb-Ac* and *pk2Ac* were subjected to ELISA assay for the quantification of insecticidal protein *Cry1Ac* levels at every 15 days intervals. Expression of *Cry1Ac* in transgenic plants revealed that *Rb-Ac* plants were showing maximum expression of *Cry1Ac* protein and no decline in expression was observed in these plants. While in case of *pk2Ac* plants, *Cry1Ac* levels declined with the age of plants (Fig. 4a). Expression in *Rb-Ac* plants was up to 250 ng/g of fresh tissue weight even when the plant was 90 days old that conferred full protection against targeted insect pests.

Cry1Ac protein was also quantified in different plant tissues of *Rb-Ac* and *pk2Ac* plants. Plant tissue i.e. leaf, boll, square, anthers and petals were undertaken for this purpose. The results showed that leaves of both *Rb-Ac* and *pk2Ac* plants were having maximum *Cry1Ac* concentration followed by bolls and squares. *Rb-Ac* plants showed more expression of *Cry1Ac* in bolls and squares as compared to *pk2Ac* plants while no expression in anthers was recorded (Fig. 4b).

Discussions

This study was undertaken to determine the spatio temporal expression of insecticidal gene *Cry1Ac* in transgenic cotton lines. Spatio temporal studies revealed that expression level of *Cry1Ac* declined during the crop growth with toxin level falling to 15–20 ng/g of fresh tissue weight (Fig. 1a). These results were in agreement with the previous studies conducted by Fitt et al. (1998), Greenplate et al. (1998), Chen et al. (2000), Mahon et al. (2002), Xia et al. (2005), Olsen et al. (2005), Manjunatha et al. (2009) and Adamczyk et al. (2009), Bakhsh et al. (2010) and Bakhsh et al. (2011a, b) who have reported inconsistency in insecticidal gene expression over the crop growth period. Expression levels of insecticidal gene were also remained variable in different plant parts (Fig. 1b). The leaves of Bt cotton were having maximum expression as compared to certain fruiting parts (Greenplate 1999; Greenplate et al. 2000; Adamczyk et al. 2001; Gore et al. 2001). Furthermore, the results obtained from biotoxicity assays at different intervals also confirmed the variation in *Cry1Ac* expression (Fig. 2).

Gene expression varies with the nucleotide sequence of the gene, promoter, and the insertion point

Table 1 Transformation efficiency of cotton variety NIAB-846 with *Rb-Ac* and *Pk2Ac* constructs

Average transformation efficiency = 0.55%

Construct	No. of germinating seedlings used	Survival % (3–4 weeks)	Survival % (7–8 weeks)	Plants in soil	Transformation efficiency (TE %)
Rb-Ac	2000	225	50	12	0.60
Pk2Ac	2000	735	43	10	0.50

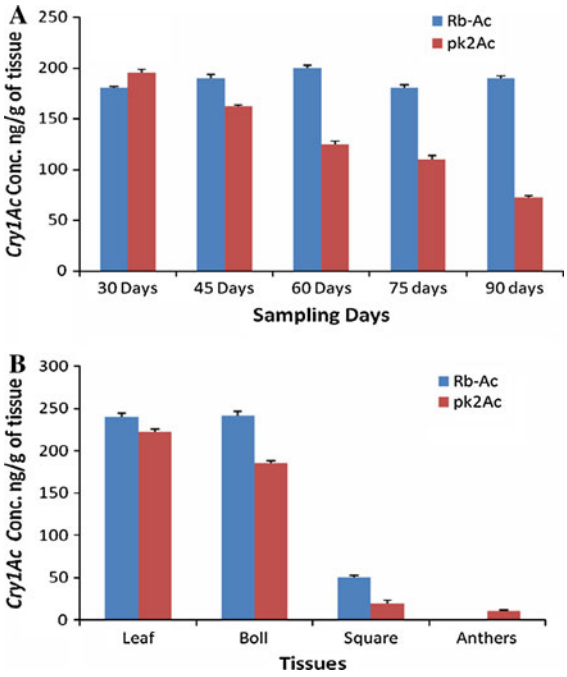

Fig. 4 Spatio-temporal expression of *Cry1Ac* in transgenic cotton plants of *Rb-Ac* and *pk2Ac*. **a** Temporal expression of *Cry1Ac* quantified showed higher and consistent expression of *Cry1Ac* in *Rb-Ac* plants while decline in expression was observed in *pk2Ac* plants. **b** Spatial expression of *Cry1Ac* quantified showed higher *Cry1Ac* levels in leaf, boll and squares in *Rb-Ac* plants as compared to *pk2Ac* plants

of the gene in the DNA of the transgenic variety, transgene copy number, the internal cell environment, as well as several external factors in the environment (Hobbs et al. 1993; Guo et al. 2001; Rao 2009). A thorough research at molecular and environmental level was carried out to understand the differential expression of insecticidal protein in transgenic cotton lines and this variation in endotoxin efficacy was attributed to promoter activity (Bakhsh 2010). Results revealed that 35S CaMV promoter is cell-type-specific and is developmentally regulated promoter (Nilsson et al. 1992; Pauk et al. 1995; Wessel Vl Tom et al. 2001; Sunilkumar et al. 2002; Haddad et al. 2002; Yang and Christou 2005; Amarasinghe et al. 2006; Bakhsh et al. 2010). The study triggered research to find possible new promoter that would induce more consistent production of insecticidal gene throughout the life of the cotton plant. For this purpose, a *rbcS* promoter from desi cotton (*G. arboreum*) was isolated.

The small subunits promoters and their transit peptides are attractive candidates for expression of genes at high levels and have shown promising expression results in driving the expression of introduced genes (Wong et al. 1992; Fujimoto et al. 1993; Data et al. 1998; Song et al. 2000; Jang et al. 2002; Liu et al. 2005; Panguluri et al. 2005; Amarasinghe et al. 2006; Anisimov et al. 2007; Suzuki et al. 2009; Kim et al. 2009 and Bakhsh et al. 2011a, b). *rbcS* promoter was cloned in a plant expression vector pCAMBIA 1301. In the present study pCAMBIA 1301 vector was used to transform as it has been used by many researchers to transform the genes like cbnA in rice (Shimizu et al. 2002), *acsA* and *acsB* genes, USP-1 and HSP-26 genes in cotton (Li et al. 2004; Maqbool et al. 2010), *HMGR* gene (3-hydroxy-3-methylglutaryl-CoA reductase) in *Taraxacum* plants (Bae et al. 2005). A local cotton cultivar NIAB-846 was transformed with *Cry1Ac* driven by *rbcS* promoter. The same cotton cultivar was also transformed with *Cry1Ac* gene driven by 35SCaMV promoter to compare the expression pattern of insecticidal gene under two different promoters.

In the present study, agrobacterium-mediated transformation procedure established by Gould and Magallanes-Cedeno (1998) was followed with some modifications adopted at CEMB as described by Rao (2009), Bakhsh (2010) and Khan et al. (2011). The transformed plants were analyzed for DNA integration and expression through PCR and SDS-PAGE (Fig. 3).

The titer of *Cry1Ac* toxin in newly transformed *Rb-Ac* and *pk2Ac* plants quantified at different developmental stages indicated clearly that *Rb-Ac* plants were expressing *Cry1Ac* toxin at high concentration as compared to *pk2Ac* plants. The recorded data showed that *rbcS* is efficient promoter driving the expression of *Cry1Ac* consistent in cotton plant while a decline in *Cry1Ac* was observed in *pk2Ac* plants (Fig. 4a). Spatial expression of *Cry1Ac* gene in different plant parts further confirmed that promoter activity is limited to green parts of plants i.e., leaf, stems, and square buds. The high expression of *Cry1Ac* protein was quantified in *Rb-Ac* plants being maximum in leaf as compared to *pk2Ac* plants that were showing less concentration of *Cry1Ac* (Fig. 4b). Expression of insecticidal protein is most critical in leaves as majority of the newly hatched lepidopterans larvae initially feed by scraping chlorophyll in the tender leaves and, as they grow, moves over to the squares and bolls for further feeding and development (Jayaraj 1982; Gore et al. 2002).

The variability in expression of insecticidal gene was recorded in plants having *Cry1Ac* under *rbcS* promoter that induced consistent *Cry1Ac* expression at different growth stages of cotton plant. The use of this promoter is also useful for addressing the biosafety issues as this promoter activity is limited to green parts of plants, hence no gene expression in roots and cotton seed was observed (data not shown), ultimately no threat to soil organisms and safety of cotton products and by products can also be assured.

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