The cowpea trypsin inhibitor promoter drives expression in response to cellular maturation in *Arabidopsis thaliana*

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ABSTRACT

The Bowman-birk type trypsin inhibitors accumulate in high concentration in legume and cereal seeds, especially during seed maturation and are considered to be involved in insect tolerance. The 5' flanking sequences of the trypsin inhibitor was isolated from cowpea genomic DNA using anchor PCR. Analysis of sequences showed presence of seed specific RY elements and also other elements associated with seed development such as abscisic acid responsive elements (ABA responsive elements; ABRE) and dehydration responsive elements (DRE). Spatial and temporal control of the promoter driven expression pattern was analyzed using *gus* as reporter. Expression was found to occur both in embryo and endosperm; starting from torpedo stage of embryogenesis and continuing till the stage of final maturation *i.e.* bent cotyledon stage. Additional expression analyses showed that the promoter actually drives expression in tissues like leaves, roots, stipules, *etc.*, but followed a specific pattern. Comparative analysis of expression in seeds and other organs indicated that the promoter driven expression is in response to cellular maturation. [Physiol. Mol. Biol. Plants 2010; 16(1) : 31-37] E-mail : jd_research@iari.res.in

Keywords : Bowman-Birk type Trypsin inhibitor; *gus* expression; Cellular maturation; Seed development; Regulation

Abbreviations : DAF – Days After Flowering, BBTI – Bowman-Birk type Trypsin Inhibitor, ABA – Abscissic acid, ABRE – ABA Responsive Elements

INTRODUCTION

Bowman-birk type protease inhibitors (BBTIs) are cysteine-rich proteins with inhibitory activity against proteases that are widely distributed in monocotyledonous and dicotyledonous species (Lin *et al.*, 2006). BBTIs from dicotyledonous seeds are double-headed, with two homologous domains; each bearing a separate reactive site for the cognate proteases. These domains interact independently, but simultaneously with two proteases which may be same or different. The first reactive site in these inhibitors is usually specific for trypsin, chymotrypsin and elastase (Qi *et al.*, 2005). The canonical inhibitory loop of the domain contains 11 amino acids (Jaulent and Leatherbarrow, 2004) which by itself is sufficient to inhibit the proteases. Presence of cysteine residues in the domain is known to be important for its inhibitory role, which possibly provides stability to the structure.

BBTIs accumulate heavily in storage organs like seeds of legumes and cereals during seed maturation and also in tubers (Hilder *et al.*, 1989). These inhibitors contribute heavily to the cysteine fraction in legume seed proteins and their proposed function is in storage. They have also been attributed with functions like those of dehydrins and as deterrents against herbivory (Koiwa *et al.*, 1997) and pathogenic infections. But their possible role in regulation of protein accumulation and hydrolysis remains doubtful as an endogenous target is yet to be identified.

Most studies on BBTIs point to its deterrent role against insect pests. However, the regulation of expression of these proteins is not very clear. The presence of many isoforms makes the study quite complicated. Proteomic studies in seeds of barley and *Medicago* indicates that their accumulation start from early phase of seed development and increases up to the final stage of seed development (Finnie *et al.*, 2002; Gallardo *et al.*, 2003). Thus these proteins could be under the direct control of developmental signals for

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seed development and may be regulated by factors like ABA, dehydration, *etc* which are important during seed maturation.

The protein and gene sequences of Bowman-birk type trypsin inhibitor has previously been isolated from cowpea (*Vigna unguiculata*) which confers enhanced insect resistance to transgenic tobacco plants (Hilder *et al.*, 1989). Self-association tendency is commonly observed in BBTIs. The BBTI from *Vigna unguiculata* (Vu-BBTI) also has been found to undergo self-association to form very stable dimers and more complex oligomers. The exposed hydrophobic surface patches in the monomer and the strong hydrogen bonded network in the dimer structure explains the reason for BBTIs to exist in dimeric as well as other multimeric forms (Rao and Suresh, 2007).

In the present study, 5' upstream regulatory elements of trypsin inhibitor have been isolated from cowpea (*Vigna unguiculata* L.). The promoter activity was tested by performing reporter analysis with 'gus' as reporter in transgenic *Arabidopsis thaliana*.

MATERIALS AND METHODS

Plant Material

The seeds of cowpea (*Vigna unguiculata* L.) var V130 were obtained from Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi. The cowpea plants were grown in field during the *Kharif* season (July–September). *Arabidopsis* plants were grown in controlled growth chambers at the National Phytotron Facility, IARI. The plants were grown at 22 °C and 70–80 % relative humidity with 16 hrs and 8 hrs of light and dark cycles respectively.

Expression analysis of cowpea trypsin inhibitor

Total RNA was isolated from various plant tissues including leaf, root, stem and seed, and then treated with RNase-free DNase prior to the reverse transcription. RT-PCR analysis was carried out using one step RT-PCR kit (Qiagen) according to manufacturer's instruction. Based on the sequence of CpTI, the forward and reverse primers; Fcpti (5' GGTGCTAAAGGTGTGTGTGC 3') and Rcpti (5' TGTCTTCCCTTACACAAC 3') were designed for RT-PCR. Simultaneously, for the expression of trypsin inhibitor during seed development, pods from field grown plants were harvested at 5-day interval, starting from 10th day after flowering (DAF) till maturity and

total RNA was isolated from seeds at different stages of development.

Walker PCR for promoter amplification

Genomic DNA was isolated using DNeasy kit from Qiagen. The trypsin gene was amplified using the primer pair (5'GGTGCTAAAGGTGTGTGTGC3', 5'TGTGTTCCCTTACACAACAAACA3'). The amplification was carried out for 25 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The amplified gene sequence was used for designing primers for walker PCR.

The anchor PCR protocol was followed for the promoter isolation. Total genomic DNA was digested with the four enzymes *Bam*HI, *Bgl*II, *Bcl*I and *Sau*3A. The overhangs were filled with dATP and dGTP using Klenow fragment and were ligated with adapter sequence. This product was used as template for amplification using T7 primer and R1 (5'TCAAATCCATGGCTGCAGTA3') and the second nested amplification was done with T7 primer and R2 (5' CTTCCGAGGTGGTTCAAATC3'). Both reverse primers (R1 and R2) were chosen to be near to the transcription start site to amplify the maximum possible flanking region.

Promoter-reporter fusion vector construction and transformation

The promoter-reporter vector was constructed using pCAMBIA1305.1 as template by replacing the CaMV-35S promoter with the isolated promoter using *Eco*RI and *Nco*I restriction sites. The vector was mobilized to *Agrobacterium* strain GV3301 and was used to transform *Arabidopsis* by *in planta* method (Clough and Bent, 1998). The transformed plants were selected on hygromycin medium (10 mM KNO₃ + Agar + 25 mg hygromycin/ Liter). T₀ plants were selfed to produce T₁ plants in order to analyze segregation and this was continued for T₂ and T₃ for homozygosity. The plants derived from different transformation events were considered as different T-DNA insertions and T₁ population showing 3:1 segregation were considered as single T-DNA insertions.

Sequence Analysis

Sequence analysis for similarity was carried out with NCBI BLAST (www.ncbi.nlm.nih.gov) and sequence analysis of the promoter for the presence of *cis* acting elements was carried out using PLACE database (Higo *et al.*, 1999).

GUS histochemical assay

For whole mount staining, the plant tissue was incubated overnight in a buffer containing 0.5 mM X-Gluc and destained using 70 % ethanol (Jefferson *et al.*, 1987). Seeds were cleared in modified Hoyer's medium (Stangeland and Salehian, 2002) after destaining.

ABA induction studies

The roots of transformed *Arabidopsis* plants were incubated in MS medium containing 5 μ M ABA (considered as biological concentration) for one hour and were stained for GUS expression.

RESULTS

Detection of cowpea trypsin inhibitor transcripts in tissues other than seeds

The cowpea protease inhibitor (CpTI) gene was amplified based on the sequence available from database (Accession number X51617). The inhibitor belonged to Bowman-Birk type serine protease inhibitor, which is commonly present in legumes and cereal seeds (Koiwa *et al.*, 1997). The inhibitor coded for a double-headed inhibitor, which inhibits both trypsin and chymotrypsin. The relative quantity of CpTI mRNA in the seeds seemed clearly dependent on the developmental stage. The expression studies using RT-PCR detected an amplicon of 280 bp in seed, leaf, root and stem indicated that probably trypsin inhibitor is indeed constitutively expressed (Fig. 1A). The amplicon intensity was higher in 10th DAF seeds, decreasing throughout seed development; the lowest being on 35th DAF (Fig. 1B). As trypsin inhibitor genes are usually present in many isoforms, the detection of transcripts could be due to the expression of similar genes in different tissues or a single gene is being expressed in all tissues. A study of

5' upstream elements was expected to establish the expression conclusively. So the promoter for cowpea trypsin inhibitor was cloned in an effort to characterize the expression pattern.

Cloning of 5' upstream sequences and sequence analysis

A 600 bp fragment was amplified using *Bgl*II digested genome walker library. The transcription start site (TSS) for the promoter was mapped using 5' RACE and it was found to be present 45 bp upstream from start codon. Sequences mapped after transcription start site were analyzed for the presence of probable 'cis' acting elements using the PLACE database. Analysis revealed the presence of common motifs of promoter like TATA (-25 bp) and CAAT boxes (-80 bp). The promoter is known to contain elements like RY repeat, which is specific for seed specific promoters (Fujiwara *et al.*, 2002). RY repeat was in fact present at a distance of 100 bp from the TATA box. Presence of RY repeat near TATA box is known to be an important feature of seed specific promoters (Fig. 2). Apart from this, RY repeat is a known target for many transcription factors like ABI3 and FUS3 of *Arabidopsis* (Bobb *et al.*, 1997), PvALF of French bean (Reidt *et al.*, 2000) and VP1 of maize (Suzuki *et al.*, 1997) which are the main factors involved in seed maturation. In addition, other elements like ABA sensitive and dehydration specific elements were also present. These elements are known to act in conjunction with other elements, similar to ABRE interacting with RY repeat in *napA* promoter of *Brassica* to regulate expression in a seed specific manner (Ezcurra *et al.*, 1999). ABRE being ubiquitous, its interaction with other elements might decide the tissue specific expression (Nambara and Marion-Poll, 2003) and seed specific activation might be one of them. Usually, seed specific promoters are characterized by clustering of 'cis' acting elements near to transcription start site (Bobb

Fig. 1. (a) Expression analysis of cowpea trypsin inhibitor transcripts in different tissues of cowpea, Lane M. 100bp DNA ladder, Lane 1, plasmid control; Lanes 2-5. RNA from: 2, seeds; 3, stem; 4, root; 5, leaves (b) RT-PCR analysis in developing seeds of cowpea, Lane M. 100bp DNA ladder, Lanes 1-6. Seeds from: 1, 35 DAF; 2, 30 DAF; 3, 25 DAF; 4, 20 DAF; 5, 15 DAF; 6, 10 DAF.

et al., 1997; Chen *et al.*, 1986; Shirsat *et al.*, 1989). Similar clustering of elements within 200 bp from TSS was observed in the isolated promoter (Fig. 2). These features suggest the possible seed specific expression of the promoter.

Spatial and temporal regulation of promoter driven *gus* expression in seeds of transgenic *Arabidopsis*

In order to check for expression driven by the promoter, promoter-reporter analysis was carried out. Reporter analysis was done in stable transformants of *Arabidopsis* using *gus* as a reporter. *gus* expression was found in all organ types, but they appeared to follow specific patterns in response to some developmental cues. Expression in seeds was found in all tissues *i.e.* embryonic axis, cotyledons and endosperm, but not in the testa. The expression started from late torpedo stage of embryogenesis and continued till the end of maturation through straight cotyledon and bent cotyledon stages (Fig. 3A-D). A similar pattern of *gus* expression was reported using 2S1 storage protein of *A. thaliana* promoter (Devic *et al.*, 1996). The expression of the isolated promoter happened early in seed maturation phase when compared to globulins, which are detected at later stages of cotyledon development (Devic *et al.*, 1996; Heath *et al.*, 1986). This result is also in agreement with proteomic analysis in seeds of *Medicago* and barley where protease inhibitors were detected at early stages of maturation and continued till the end of maturation (Finnie *et al.*, 2002; Gallardo *et al.*, 2003).

Expression of GUS in tissues other than seeds

Apart from seeds, *gus* expression was detected in other parts of the plant as well. In roots, the expression was confined to the elongation zone from the tip of all roots, including tap root and lateral roots. Figure 3I shows

that GUS staining had occurred in all layers of root; even in the root hairs located in the region. Then the expression in roots moved away from outer layers to central axis and finally expression was found to be completely absent in mature parts of roots. Expression in other parts like leaves and stipules also showed specific pattern. The young leaves were not stained, but lower leaves showed *gus* expression, it receded from the tip of leaf to base (Fig. 3E) and was absent in mature leaves (Fig. 3F). GUS staining in stipules occurred earlier than that in leaves. Figure 3 (G & H) shows *gus* expression to be present in stipules and not in leaves. This differential expression may be due to differential developmental states of the two parts.

ABA induction studies in roots of transgenic *Arabidopsis* plants

Since roots show the different phases of development very distinctly, ABA induction was carried out in roots of transformed *Arabidopsis* plants. ABA induction does not cause induction of expression in all tissues, but ABA induction resulted in extension of GUS activity to the meristematic zone (Fig. 3J).

DISCUSSION

The results show that expression pattern of a major seed protein, cowpea trypsin inhibitor (CpTI), does not confine to seeds alone. The expression also extends to other tissues, following a specific pattern. Qureshi *et al.* (2007) also reported similar expression pattern of another storage protein (lectin) in tissues and developing seeds of chickpea (*Cicer arietinum*) by using RT-PCR. Though the possible function could not be assigned conclusively, it implies the possibility of having a multifunctional role.

Fig. 2. Schematic diagram showing the 'cis' acting elements predicted at PLACE database, indicating the clustering of elements near TATA box.

Fig. 3. Gus expression analysis in reporter transformed *Arabidopsis*. (a) Whole seed gus staining showing expression in embryo, endosperm, but not in seed coat, (b) Late torpedo stage, expression in all parts of embryo like cotyledon, radicular pole, (c) Straight cotyledon stage, (d) Bent cotyledon stage, (e) Immature leaf showing expression that moves from tip to downwards, (f) Matured leaf – expression is highly dispersed and about to disappear, (g) Young leaf showing expression in stipule (pointing arrow), (h) Stained stipule of G, (i) Root tip –expression in elongation zone and getting reduced towards matured tissues, (j) ABA induced root, expression seen in whole root tip

Gus expression driven by the CpTI promoter in *A. thaliana* provides insights into the developmental regulation of CpTI gene. Developmental regulation of the CpTI by seed maturation process as understood from this study is in agreement with proteomic studies in *Medicago* and barley having typical seed accumulated serine protease inhibitors. Expression starts in late torpedo stage and continues through straight and bent cotyledon stages. But the expression in tissues of leaves, stipules, roots, *etc* indicates possible function in tissues other than those of the seeds as well. *Gus* expression driven by promoter firmly establishes that the expression pattern is specific and it follows developmental cues. The expression specifically detected in maturing tissues and the pattern of retreat from the tip downwards is typical to leaf developmental event (Poethig, 1984).

In order to have a clear idea about expression, the expression in seeds and other tissues were comparatively analyzed. Expression driven by isolated promoter begins at torpedo stage of embryogenesis. This stage coincides with the beginning of embryonic growth arrest (Raz *et al.*, 2001). The expression in roots also follows a similar pattern, but there is spatial separation of events. The expression was confined to elongation phase next (after) to the meristematic zone. Further, the genes (ABI3, LEC1) which control maturation event in *Arabidopsis* seeds are not only confined to seeds, but also extend to vegetative quiescence and rejuvenation of chloroplast after etiolation (Lotan *et al.*, 1998; Rohde *et al.*, 2002). Similarly, the ABI3 homolog in poplar is involved in growth arrest and dormancy of buds (Rohde *et al.*, 1999). Apart from these regulatory genes, the expression of 2S1 gene of *Arabidopsis* during quiescence and accumulation of storage proteins of dormant bud of *Populus* indicates the possible expression of seed proteins in other tissues. However, CpTI expression starts early in seed maturation compared to other seed storage proteins and is seen in developing tissues as well as meristematic tissues. Thus regulation may be different from late stage seed proteins but it matches with expression (or their domain of influence) of regulatory genes like ABI3/LEC1. Therefore, we hypothesize that the isolated promoter is under the control of some factors which function commonly during growth arrest.

Analysis of expression from the view of plant development would give us a better understanding. Plant development is continuous and the embryogenesis and adult plant development cannot be considered as individual processes (Kaplan and Cooke, 1997). Developmental processes continue even after

embryogenesis. Therefore, maturation is a common process during which cells undergo a preparation before differentiation. A similar process of maturation (or cellular maturation) has been proposed by Bonetta and McCourt (1998). Expression in seeds and other tissues in specific pattern indicate that the gene expression may be regulated according to cellular maturation process. To confirm this hypothesis, ABA induction analysis was carried out. Since roots show the phases very distinctly, assay was carried out in root. ABA induction resulted in extension of GUS activity to the meristematic zone (Fig. 3J). ABA is known to induce growth arrest (embryonic and vegetative quiescence). Extension of expression to meristematic zone confirmed that the expression is specific to growth arrest. The stipules showed *gus* expression earlier than leaves, but followed a similar pattern. But there was a mismatch in time of expression between leaves and stipules (Fig. 3G & H). Thus comparative analysis of expression between different tissues gave insight into the developmental regulation of CpTI driven expression and its possible multifunctional role.

The results of present study provide insights into plant developmental processes. Sequential but spatial separation of the developmental processes (*i.e.* the meristematic activity, cell maturation/growth arrest and differentiation) makes plant development a continuum. Similarly, bud dormancy and seed maturation can be considered as altered developmental programs of plants in response to developmental stimuli and/or environmental cues. Similarity between bud and seed dormancy implies that seed dormancy may be an elaboration of an already available maturation process. So the isolated promoter can be considered to be active during maturation process. Profuse expression in seeds may be due to extended period of maturation. This might have resulted in acquisition of storage like function by the protease inhibitor genes.

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