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Inheritance and expression of *Cry2Aa* gene in transgenic chickpea (*Cicer arietinum* L.)

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Abstract

The transmission and expression of *Cry2Aa* gene driven by *Arabidopsis* Rubisco small sub unit gene (*atsA1*) promoter (AraSSU) in chickpea was previously analysed in the T_1 generation of 13 independent transgenic lines. It is desirable that transmission of transgene(s) by maternal and paternal parents be consistent and predictable. We selected three representative lines, BS5A, BS6H, and BS6L to study transgene transmission and expression up to the T_6 generation. The line BS5A has tightly linked multiple transgene inserts, while lines BS6H and BS6L appeared to transmit one segregating transgenic insert to their progeny. The *Cry2Aa* gene was inherited up to the T_6 generation in all three lines tested. In the case of line BS5A, the level of expression of Cry2Aa protein varied within the T_1 progeny. The expression of the Cry2Aa protein was found high in progeny of the lines, BS6H and BS6L and the lines showed stable and predictable expression of Cry2Aa protein up to T_6 generation. Surprisingly, we have been able to identify homozygous progeny in line BS5A, while lines BS6H and BS6L are segregating even in the T_6 generation. Loss or silencing of *Cry2Aa* gene under the control of AraSSU promoter was not observed and the transgene inherited and expressed stably in chickpea.

Key words: Transgene, *Cry2Aa*, chickpea, inheritance, expression

Introduction

The important applications of genetic transformation are to incorporate agronomically important new gene(s) or modify native gene of crop plants and expedite crop breeding programme. However, successful application of gene technology depends on stable and predictable inheritance and expression of transgene(s). Transgene(s) could be transmitted predictably to sexual progeny and these progeny may show meiotic and expression stability [1-3]. There are instances where the transgene inheritance and expression is affected by various factors such as number of transgene copy inserted in locus or in different loci, site of integration and orientation of the transgene; and silencing of transgene in the progeny of the transgenic lines [4-7]. Therefore, it is important to know the inheritance and expression pattern of the transgenic lines before incorporating transgenic lines in a breeding programme.

Development of transgenic crop is now feasible in *in vitro* recalcitrant crop such as chickpea (*Cicer arietinum*) using gene technology methods. Using genetic transformation procedure transgenic chickpea were developed using various Bt-*Cry* genes for resistance to one of major pests *Helicoverpa arimegra* [8-11]. In our laboratory transgenic chickpea lines were generated using a codon-optimized *Cry2Aa* gene. The transmission and expression of the *Cry2Aa* gene was tested in T_1 generation in 13 independent transgenic lines. The lines expressing Cry2Aa endotoxin in high levels were resistant to *Helicoverpa* larvae in insect bioassays [11]. However, inheritance and expression of *Cry2Aa* gene in successive generations have not yet been studied. In the transgenic plants instability of the transgene(s) occurs frequently [12, 13]. The mechanisms of this instability are not fully understood, however, transgene methylation, number of transgene copies per genome, rearrangement of transgene insert, site of insertion in genome and homology of endogenous gene to transgene, are responsible for transgene expression instability [14-21]. Therefore, we studied inheritance and expression of *Cry2Aa* gene in Bt chickpea and found it to be stably inherited and expressed in advanced generations.

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Materials and methods

Three transgenic chickpea lines, BS5A, BS6H and BS6L developed using a chimeric *Cry2Aa* gene by *Agrobacterium* mediated method were selected for the present study. Genomic DNA was extracted from the young leaves of the progeny of the transgenic lines as well as untransformed chickpea plant as per Doyle and Doyle [22]. PCR analyses were performed to exhibit presence of transgene in the transgenic plants using the forward primer: 5'-TGTCCATCTGGTCCCTCTTC-3' and the reverse primer 5'-ATGGTGAAGCCGGT GAGTC-3' to obtain a 720 bp amplicon from the coding region of the *Cry2Aa* gene. For the amplification of the *Npt*II gene another set of primers (forward: 5'-GGTGGAGAGGCTA TTCGGCTA-3' and reverse: 5'-GGTAGCCAACGC TATGTCCTGA-3' amplifying a 649 bp fragment was used. PCR was carried out using standard protocol and the amplified products were electrophoresed on 1.5% agarose gel.

For Western and Dot blot analyses, total protein was extracted from young leaves in extraction buffer containing 1M Tris-Cl; (pH 8.0), 1 M NaCl and 2mM EDTA, and 1mM PMSF. Protein concentration was determined by the method of Bradford [23]. On each gel 30 μg of protein was loaded and electrophpresed in 10% SDS PAGE gels for Western blot analysis. The proteins from the gel were transferred to a nitrocellulose membrane by electroblotting technique. For dot blot analysis, 5μl of protein was blotted on to nitrocellulose membrane. Each blot was treated with primary antibody raised in rabbits against Cry2Aa inclusion bodies and alkaline phosphatase conjugated antibody raised in goats against rabbit IgG was used for detection. The blot was developed in 10ml of Nitro Blue Tetrazolium (NBT) and 5-Bromo-1 chloro-3-indoly phosphate (BCIP) solution (Sigma) for 15-20 minutes and the reaction was stopped by washing the membrane with distilled water.

Results and discussion

To incorporate the transgenic crops in a breeding programme, it is imperative that transgene(s) be stably expressed and inherited without silencing in successive generations. Therefore, stability of transgene expression and inheritance was studied in transgenic chickpea lines containing Bt-*Cry2Aa* gene. Thirteen independent transgenic lines were produced by *Agrobacterium* mediated method. Independent lines were previously confirmed by Southern blot hybridization [11]. The southern blot analysis confirmed integration of multiple copies in the line BS5A, while single copy T-DNA insertion was observed in the lines, BS6H and BS6L. The line BS5A showed a typical 3:1 ratio for transgene segregation in T_1 generation. The multiple copies of the transgene appeared to be tightly linked and co-segregated in the successive generations. In the T_1 generation the lines, BS6H and BS6L, showed transgene segregation in a Mendelian 3: 1 ratio. The line BS5A was found to express Cry2Aa endotoxin at a low level and conferred only 30% mortality of *Helicoverpa* larvae while the lines BS6H and BS6L, are high expressors and showed 98% larval mortality in insect bioassays [11].

Transgene segregation in advanced generations

The T_2 progeny of all the three selected lines were raised in the greenhouse and transgene inheritance and expression over several generations (up to T_6) was carried out using PCR and western/dot blot techniques. In the T_2 progeny of the plant BS5A.14 displayed 3:1 ratio when subjected to PCR followed by western blot (Fig. 1a) analyses and homozygous progeny (Fig. 1b) were indentified in the T_6 generation. In the case of lines, BS6H and BS6L a stable Mendelian segregation of single insert was observed up to T_6 generation (Table 1).

Transgene expression, inheritance and silencing have been described in many transgenic plants and their progeny [24]. In transgenic wheat transformed with *uidA* and *bar*, only one out of six transgenic lines tested expressed GUS in the T_2 generation, while the *bar* gene was stably expressed in five lines [5]. In addition, transgenic maize expressing CaMV 35S or adh1 promoter driven *uidA* and *bar* were found unstable in both expression and transmission in the T_1 to T_3 generations [25-27]. In transgenic barley *bar* gene silencing and poor transgene transmission in progeny of transgenic lines was also reported [28]. In the present study, inheritance of *Cry2Aa* gene appears to be stable over the generations.

The transgene expression may vary extremely depending on factors such as site of integration, transgene silencing due to methylation or presence of multiple copies [29-32] of which the 'positional effects' play a major role. If the transgene gets inserted in transcriptionally active area, its expression may be influenced by proximal regulatory sequences [33]. In this study, the expression of transgene was unstable in the progeny of the line BS5A and variation in the level of expression of Cry2Aa protein within the progeny of the line BS5A in successive generations was observed

Table 1. Segregation of Cry2Aa gene self in the selfed progeny of three independent transgenic lines

Line	Generation	Progeny ID	Segregation ratio of Cry2Aa gene *(+ve:-ve)*
BS5A	T_1	BS5A	31:5
	T_2	BS5A 14	8:3
	T_3	BS5A.14.2	12:0
	T_4	BS5A.14.2.4	8:0
	T_5	BS5A.14.2.4.6	15:0
	T_6	BS5A.14.2.4.6.8	22:0
BS6H	T_1	BS6H	19:4
	T_2	BS6H.4	6:1
	T_3	BS6H.4.4	7:2
	T_4	BS6H.4.4.2	5:2
	T_5	BS6H.4.4.2.1	6:2
	T_6	BS6H.4.4.2.1.2	5:1
BS6L	T_1	BS6L	11:4
	T_2	BS6L.2	9:4
	T_3	BS6L.2.4	8:0
	T_4	BS6L.2.4.6	6:2
	T_5	BS6L.2.4.6.2	10:1
	T_6	BS6L.2.4.6.2.8	4:3

(Figs. 1a&b). The reasons for such variation in Cry2Aa protein expression within the progeny was not clearly understood and needs further investigation. Instability of transgene expression in lines with multiple copies of transgenes may inhibit transgene expression and even lead to transgene silencing in transgenic plants. Excision of multiple transgenic loci present in the same plant can result in undesirable loss of transgene and its expression in subsequent generations. In few instances transgenes failed to transmit to the successive generations in wheat [19] and tritordeum [34]. Cannell *et al.* [35] observed silencing or a gradual reduction in marker gene expression over three generations of transgenic wheat lines. In the case of transgenic rice transformed with the *chitinase* and *hpt* genes driven by CaMV 35S promoter transgene silencing was observed in T_3 and T_4 generations [36]. Northern blot analysis showed that this silencing occurred at the transcriptional level. Similarly, transgenic wheat, expressing GUS expression under the control of the rice actin promoter showed transgene silencing due to DNA methylation [37]. Therefore, aforementioned factors may be the cause of variation in the level of expression within the progeny of BS5A which needs confirmation.

In the present study the lines BS6H and BS6L, exhibited stable transgene inheritance and expression in the successive sexual generations (Figs. 2a&b). Kihara *et al*. [38] observed expression of the thermostable b-amylase gene driven by the seed-specific b-amylase promoter was stably transmitted up to the T_4 generation. Similarly, recombinant proteins thioredoxin h driven by the B1-hordein promoter was transmitted up to the T_3 generation [39] and heat stable β-glucanase driven by the D-hordein promoter in T_1 seed [40] was recently described. Horvath *et al*. [41] also showed that stability of transgene expression of heat stable β-glucanase driven by an aleurone-specific promoter occurred in two out of three primary T_0 transgenic lines tested up to the T_5 generation. Recently, stable transmission of low copy number transgene (*gus* and *bar*, and *1Ax1* and *bar*) was observed in commercial wheat [42].

Although we observed that the transgene inheritance and expression was stable over successive generations in the lines, BS6H and BS6L, but homozygous progeny were not obtained even in the T_6 generation. It appears that high level of expression may be lethal in obtaining homozygous progeny in these lines. Transgenic rice expressing a high level (>1% of total soluble protein) of Cry1Ac and Cry2A protein showed developmental and morphological defects such as stunted growth and sterility [43]. We also observed reduction of plant phenotype in plants with high expression of Bt-*Cry2Aa* gene [11]. Therefore, it may be possible that the homozygous embryos are aborted due lethal effect of transgene or it is possible that the putative homozygous progeny did not survive till maturity as we observed stunted plant growth, poor seed set, premature death of seedling in the high expressing lines, BS6H and BS6L.

Thus, we observed a stable inheritance and expression of *Cry2Aa* gene driven by Arabidopsis Rubisco small subunit gene promoter in chickpea indicating possibility of such lines using in chickpea improvement program.

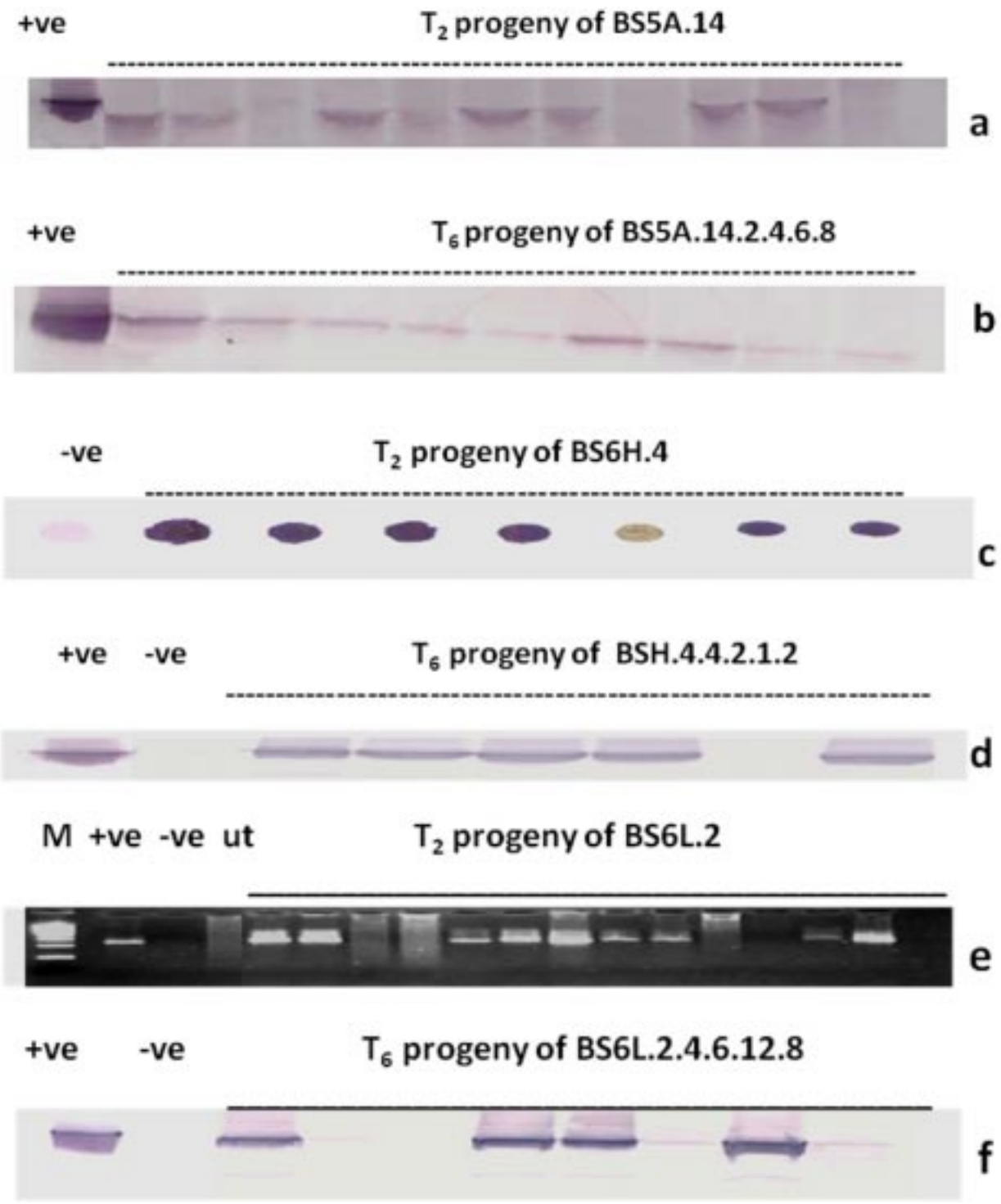

Fig. 1. Expression in the Cry2Aa protein in the T_2 and T_6 progeny of in the lines BS5A (a, b), BS6H (c, d) and BS 6L (e, f). 1a. Lane 1: Positive control (Bt Cowpea); 2-12: T_2 progeny of the line BS5A; 1b. Lane 1: Positive control (Bt Cowpea); 2-9: T_6 progeny of the line BS5A; 1c. Lane1: Untransformed chickpea; 2-7: T_2 progeny of line BS6H; 1d. Lane1: Positive control (Bt Cowpea); Lane 2; Untransformed chickpea 3-8: T_6 progeny of line BS6H; 1e. Lane 1: Marker; 2: Positive control (plamid, pBK201); 3: negative control; 4: Untransformed chickpea; 5-17: T_2 progeny of line BS6L; 1f. Lane1: Positive control (Bt Cowpea); Lane 2; Untransformed chickpea 3-10: T_6 progeny of line BS6L

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