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# Introgression of Transgenic Event in a Molecular Breeding Variety for Pyramiding Resistance to *Fusarium* wilts and Pod Borer in Chickpea (*Cicer arietinum* L.)

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**ABSTRACT**

Chickpea (*Cicer arietinum* L.) is one of the most cultivating pulse crops globally and in India, its productivity is limited blisteringly by Gram pod borer insect (*Helicoverpa armigera*) and *Fusarium* wilt disease. Pyramiding of these two biotic stress resistance in a single genotype is expected to increase crop productivity and reduces the usage of pesticides/fungicides by farmers, which boost economic viability in the cultivation of this crop. Hence, we planned to transfer the *cry1Ac* gene, which imparts pod borer resistance from BS 100B event, to Super Annigeri-1(SA-1), the wilt resistant variety bred through marker-assisted backcrossing.The $F_1$ plants developed by SA-1 X BS 100B crosses were confirmed with *cry1Ac* gene-specific marker and polymorphic SSR marker (ICCM0299).The expression of the *cry1Ac* gene at the transcriptional level through reverse transcription polymerase chain reaction and at the protein level through enzyme-linked immuno sorbent assay was confirmed in $F_1$. The presence of a single copy of a gene integration, stably, was confirmed through the inheritance of the *cry1Ac* gene and 3:1 segregation ratio in $F_2$ using Chi-square test. BS 100B (donor parent), $F_1$ and $F_2$ plants, respectively recorded 21.47 µg, 20.43 µg and 15.31-21.17µg of *Cry1Ac* protein/g of leaf tissue in quantitative ELISA test that is enough to record pest resistance. This is the first study to combine resistance to both pod-borer and *Fusarium* wilt by intercrossing of one *cry1Ac*event (BS 100B) together with SA-1 developed through molecular breeding and developed progenies have shown resistance to both biotic stresses.

## Introduction

Chickpea (*Cicer arietinum* L.) is predominantly cultivating winter season grain legume crop in the world and secures the title of the third most important pulse crop globally for its production. India is the largest producer and contributes about 71.95 percent of global production (Thudi *et al.,* 2017). Due to its increased demand by human consumption and its ability to fix atmospheric nitrogen into the soil, it has become very popular crop among the farming and consumer communities. The potential productivity of chickpea is nearly 2.0-2.2 tones per hectare, but farmers are harvesting

only < 1 ton per hectare, less by almost 50% of the potential yield due to biotic and abiotic stresses. Among biotic stresses, *Fusarium* wilt disease and gram pod borer affect chickpea production significantly. *Fusarium* wilt is the most severe disease in the semiarid tropics where the cultivating season is too warm and dry. It is caused by *Fusarium oxysporum fsp. ciceri*, soil-borne pathogenic fungus and differs in pathogenic variability (Jendoubi *et al.,* 2017). Chlorosis, premature leaf drop, necrosis, stunting, and browning of vascular tissues are major symptoms while the losses due to wilt are estimated at around 600-750 thousand tones accounting for 20-25 percent of total global losses (Gaur *et al.,* 2012). The presence of genetic variability for wilt resistance has helped in developing wilt resistant variety through molecular backcross breeding technique. Annigeri-1 released in the 1950s is a very popular variety among farmers even today because of its wider adaptability. But it is extremely susceptible to *fusarium* wilt disease and pod borer. By using molecular breeding approach, Annigeri-1 has been converted into wilt resistant variety by collaborative efforts of UAS-Raichur and ICRISAT (Mannur *et al.,* 2019). An improved version with Super Annigeri-1 has been released in India (Mannur *et al.,* 2019).

Gram pod borer (*Helicoverpa armigera*) is a key pest due to its high rate of reproduction, short life span, polyphagous, and vigorous feeding nature (Dhingra *et al.,* 2003). Annually about 150-200 million tons of grain loss globally is due to the incidence of pod borer. Based on the severity of the infestation, the estimated yield loss varies around 10-90 percent (Manjunath *et al.,* 1989). Due to the lack of natural variation for pod borer resistance in chickpea gene pool, the success of breeding for pod borer resistance is not to the tune of required level and it is true with cotton also. In the context of chickpea, Assam Agricultural University, Jorhat successfully produced *Bt* chickpea resistant to pod borer. One of these events, BS 100 B showed 21.47 µg/g tissue expression of *Cry1Ac* protein. Therefore, due to the availability of successful *Bt* chickpea event BS 100 B and wilt resistant Super Annigeri-1 variety through molecular breeding, the present study was initiated to combine *Fusarium* wilt and pod borer resistance in Chickpea.

## Materials and Methods

### Plant material and generation of $f_1$ and $f_2$ population

Super Annigeri-1, *Fusarium* wilt disease resistant version of widely adapted Annigeri-1 but susceptible to pod borer (*Helicoverpa armigera*, Hubner) developed by University of Agricultural Sciences, Raichur and ICRISAT (Mannur *et al.,* 2019) was used as recipient parent. For pod borer resistance, chickpea *Bt* event, BS 100B (carrying *cry1Ac* gene), developed at Assam Agricultural University, Jorhat was used as donor parent. The schematic representation of the workflow is given in the Plate 1.

Artificial hybridization technique was used for generation of $F_1$ seeds from the cross between Super Annigeri-1 as recipient (♀) and *Bt cry1Ac* event (BS 100B; ♂) as donor parents. The $F_1$s were tested for hybridity by using *cry1Ac* gene specific marker and polymorphic SSR marker, ICCM0299. In $F_2$ generation, inheritance of *cry1Ac* gene and its expression was studied at College of Agriculture Vijayapura, Karnataka.

### DNA isolation and marker analysis

Genomic DNA was isolated from fresh young leaf tissues collected from 20-days old seedlings of parental genotypes, $F_1$ and $F_2$ plants through cetyl trimethyl ammonium bromide (CTAB) extraction method. The integrity of DNA was checked on 0.8% agarose gel, further quality and quantity was reconfirmed using a spectrophotometer at ODs of 260 and 280 nm. A total of 117 SSR markers evenly distributed over the chickpea genome, 12 wilt resistant linked markers (Mannur *et al.,* 2016; Varshney *et al.,* 2014a), one each *cry1Ac* and NPT II (Assam Agricultural Agriculture University,

Assam) specific markers were synthesized from Sigma Pvt Ltd. Details of these marker is given in Supplementary File 1. Polymerase Chain Reaction (PCR) amplified products were separated on 3% agarose gel and in advanced automatic capillary electrophoresis (QIAxcel Advanced, QIAxcel DNA High Resolution Kit). PCR reaction mixture contained 1 µl of sample DNA, 2 µl of 2.5 mM, DNTPs, 0.5 µl of each forward and reverse primers, 1 unit of Taq polymerase, 2 µl of 10X PCR buffer with MgCl2. Amplification cycle comprised of initial denaturation for 5 min at 94 ºC; 30 cycles of 94 ºC for 45 sec, annealing depending on primers used for 50 sec and extension at 72 ºC for 1 min.; followed by a final extension at 72 ºC for 7 min. in Master Cycler Gradient.

## Expression of cry1ac gene at transcriptional and translational level

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was performed with 30 ng total RNA isolated from 100 mg fresh leaves of 30-days old plants, using Spectrum™ Plant Total RNA Kit – Sigma. Reagents and procedure were used as per the manufacturer instructions. The quantity and quality of total RNA was determined by using spectrophotometer (Nanodrop™, Thermo Scientific) and 1.2 % formaldehyde gel electrophoresis. cDNA was synthesized by using Superscript® III first-strand synthesis kit (Invitrogen by life Technologies) according to the manufacturer's protocol. RT-PCR was carried out with above mentioned standard PCR method followed. DesiGen QL96 and Quan-T ELISA Kits for detection and quantification of *Cry1Ac* protein were used. The DesiGen 96-well works on principle of Enzyme-Linked Immuno Sorbent Assay (ELISA). Leaf tissue extracts were added to wells, pre-coated with Anti-*Cry1Ac* antibodies. A secondary Anti-*Cry1Ac* Antibody conjugate is added to the wells after adding plant samples. Substrate was added after a wash, to detect *Cry1Ac* protein in samples through a colour reaction. In quantitative ELISA, *Cry1Ac* protein concentration was estimated after recording OD vales under ELISA reader at 450nm wavelength with following formula used for calculation using OD values.

$Concentration\ of\ Cry1AcProtien = \{(OD - Blank/Slope) \times (Extraction\ buffer\ added\ to\ samples\ /\ supernatant\ taken\ and\ diluted.)\}$ / Sample weight

## Inheritance of cry1ac gene

Seeds obtained from true $F_1$ plants derived from the cross Super Annigeri-1 × BS 100B were sown during February 2019 to raise $F_2$ population in transgenic green house at College of Agriculture, Vijayapura following all guidelines. Segregation for *cry1Ac* gene in $F_2$ was recorded through PCR test using gene specific primers. Observations were recorded by scoring for the presence/absence of an amplicon and subjected to chi-square test.

$\chi^2 = \Sigma\ (Observed - Expected.)^2\ /Expected$

## Results and Discussion

## Introgression of Bt-event in genetic background of super annigeri -1

During *rabi* (winter) season of 2017, crosses were made between Super Annigeri-1(Fig. 1 a) and BS 100B (Fig. 1 b) by artificial hybridization technique and 8 $F_1$ crossed seeds were obtained (Fig. 1 c). The $F_1$ seeds were sown in transgenic green house during *rabi* season of 2018 to generate $F_2$ seeds. Screening of 177 SSR markers on Super Annigeri-1 and BS 100B *cry1Ac* event showed 7.34% (13 markers) and 51.41% (91 markers) polymorphism on horizontal (3% agarose) gel and automatic capillary electrophoresis, respectively. The $F_1$s developed by Super Annigeri-1 (recipient) × BS 100B (donor) were tested for their hybridity through PCR using *cry1Ac* gene specific marker and a co-dominant polymorphic marker ICCM0299. The presence of 509 bp amplification targeted *cry1Ac* gene, amplified with gene specific primer and presence of co-dominant amplicons with ICCM0299 in $F_1$ confirmed true hybridity (Fig. 1 d).

## Expression of cry1ac gene at transcriptional level and translational level

The RT-PCR with total RNAs from donor parent, recipient parents and $F_1$s with *cry1Ac* gene specific primer showed the targeted 509bp fragment in donor and $F_1$ plants (Fig. 3 a). Furthermore, the concentration of *Cry1Ac* protein synthesis was estimated in parents, $F_1$ and 18 $F_2$ plants using qualitative ELISA test (Fig. 3 b). As a result, *Cry1Ac* protein per gram of leaf tissue was estimated as 21.47 µg in BS 100B (positive event and a donor parent) and 20.43 µg in $F_1$ (Super Annigeri-1 × BS 100B) plants. Furthermore, *Cry1Ac* protein varied from 15.31 to 21.18 µg per gram of leaf tissue in 15 positive $F_2$ plants (Table 1).

## Inheritance of cry1ac gene in f$_2$ population

For understanding inheritance of *cry1Ac* gene, 39 $F_2$ plants derived from Super Annigeri-1 × BS 100B raised in February 2019 were analyzed with *cry1Ac* and *NptII* gene specific primers. Based on these PCR results, $F_2$ plants were classified into two classes as *cry1Ac* positive and *cry1Ac* negative plants. Out of 39 plants analyzed, 30 plants showed *cry1Ac* and *NptII* targeted region with amplicons as 509 bp and 677 bp, respectively (Fig. 2a & b). Furthermore, these results were tested for goodness of fit with 3:1 Mendalian expected monogenic ratio. The Chi-square test confirmed this ratio as calculated $\chi^2$value, 0.07693 was less than the table $\chi^2$ value (3.841) at 5% level of significance (Table 2).

## Identification plants carrying cry1ac gene and fusarium-wilt resistance genes

A total of 12 markers linked to wilt resistance were screened for polymorphism between isogenic lines, Super Annigeri-1 (wilt resistant) and Annigeri-1 (wilt susceptible), three markers namely TS-82, TR-19 and TA-96 showed polymorphism between them (Plate 2). These markers were further checked for polymorphism between Super Annigeri-1 and *Bt* event, BS 100B, only TS-82 marker showed polymorphism with amplicon sizes of 182 and 200 bp, respectively (Plate 3). As a result, TS 82 marker was screened on the 30 $F_2$ plants confirmed with *cry1Ac* gene and four plants (8, 12, 35 & 38) showed amplicon (182 bp) for TS-82 marker, whereas the remaining 24 plants yielded 200 bp amplicon (Plate 4). Therefore, these four plants have genes for both pod borer and wilt resistance. We plan to go for advancing generations through backcrossing and homozygous and stable lines will be evaluated for pod borer and wilt resistance.

As crops are suffered simultaneously by more than one stress, managing many factors to get economically viable yields by farmers is the need of the hour. Gene pyramiding and gene stacking are not a very new concepts, but with the advent of tools of genetic engineering, molecular breeding, simultaneous genetic improvement for more than one trait and improving a single trait with many genes has been becoming more effective in terms of achieving genetic gain in short time. The concept of gene pyramiding was first introduced by Watson and Singh (1952) about 70 years ago. Gene pyramiding is defined as a method aimed at assembling multiple desirable genes from multiple parents into a single genotype. The product of a gene pyramiding program is a genotype with all the target genes. For instance, Samba Mahsuri is a medium slender grain *indica* rice variety that is very popular with farmers and consumers across India because of its high yield and excellent cooking quality. However, the variety became susceptible to several diseases and pests, including bacterial blight (BB). Collaborative efforts of scientists from CSIR-Center for Cellular & Molecular Biology and ICAR- Indian Institute of Rice Research successfully incorporated bacterial blight resistance genes *Xa5*, *Xa13* and *Xa21* in to Samba Mahsuri through molecular breeding and released a new variety as Improved Samba Mahsuri (ISM). Rekha *et al.,* (2018) pyramided blast resistance genes *Pi54* and *Pi2* into bacterial blight resistance rice variety – Improved Samba Mahsuri. They used PCR based markers for bacterial blight (*Xa5*, *Xa13* & *Xa21*) resistance and blast (*Pi54* and *Pi2*) resistant genes as foreground markers and set

of 144 parental polymorphic SSR markers for background selection and identified more than 90 percent genome recovery in $BC_2F_1$ generation. As the incidences of the pod borer and *Fusarium* wilt disease are the major limiting factors in chickpea production, pyramiding of desirable genes for desirable traits with techniques of genetic engineering and molecular breeding speed up the genetic improvement of crops simultaneously for more than one trait. In the present study, it was planned to combine pod borer and wilt resistance through molecular breeding. Super Annigeri-1, wilt resistant variety is the recipient parent for pod borer resistant trait and BS 100B Bt event carrying *cry1Ac* gene was used as the donor parent.

**Table.1** Quantitative estimation of Cry1Ac protein in parents and $F_2$

| Samples | Tissue weight (mg) | OD values @450nm | OD-B | OD/slope | Cry 1Ac protein µg/g tissue |
|---|---|---|---|---|---|
| **Positive** | 70 | 1.29 | 1.07 | 23.72 | 10.17 |
| **SA- 1** | 70 | 0.23 | 0.01 | 0.21 | 0.09 |
| **100B (Ac)** | 12.5 | 0.66 | 0.60 | 13.42 | 21.47 |
| **$F_2$ -1** | 16 | 0.78 | 0.73 | 16.14 | 20.17 |
| **$F_2$-2** | 15.4 | 0.69 | 0.63 | 14.09 | 18.29 |
| **$F_2$ -3** | 12.6 | 0.06 | 0.00 | -0.01 | -0.01 |
| **$F_2$ -4** | 16.4 | 0.62 | 0.57 | 12.56 | 15.31 |
| **$F_2$ -5** | 15 | 0.07 | 0.01 | 0.21 | 0.28 |
| **$F_2$ -6** | 14 | 0.72 | 0.67 | 14.82 | 21.18 |
| **$F_2$ -7** | 14.5 | 0.58 | 0.52 | 11.60 | 16.00 |
| **$F_2$ -8** | 15.5 | 0.69 | 0.63 | 13.99 | 18.06 |
| **$F_2$ -9** | 14.4 | 0.57 | 0.51 | 11.42 | 15.86 |
| **$F_2$ -10** | 16.2 | 0.70 | 0.65 | 14.33 | 17.69 |
| **$F_2$ -11** | 12.5 | 0.64 | 0.59 | 13.00 | 20.80 |
| **$F_2$ -12** | 14.6 | 0.70 | 0.64 | 14.31 | 19.60 |
| **$F_2$ -13** | 13 | 0.06 | 0.01 | 0.09 | 0.14 |
| **$F_2$ -14** | 13.9 | 0.67 | 0.61 | 13.57 | 19.53 |
| **$F_2$ -15** | 14.9 | 0.68 | 0.62 | 13.83 | 18.56 |
| **$F_2$ -16** | 15.1 | 0.69 | 0.64 | 14.18 | 18.78 |
| **$F_2$ -17** | 15.8 | 0.61 | 0.56 | 12.41 | 15.71 |
| **$F_2$ -18** | 16.2 | 0.64 | 0.58 | 12.87 | 15.89 |

**Table.2** The $\chi^2$ tests for *cry1Ac and Npt II* gene inheritance in Super Annigeri-1 × BS 100B cross

| ***cry1Ac*gene** | **Observed (O)** | **Expected (E)** | **(O-E)** | **$(O-E)^2$** | **$\chi^2 = (O-D)^2/E$** |
|---|---|---|---|---|---|
| **Positive** | 30 | 29.25 | 0.75 | 0.5625 | 0.019231 |
| **negative** | 9 | 9.75 | -0.75 | 0.5625 | 0.057692 |
| **total** | 39 | 39 | | $\chi^2$ = 0.05, p= 3.841 | 0.076923 |

**Fig.1** Confirmation of Hybridity through Molecular markers
a. Male parent: BS100B b. Female parent: Super Annigeri-1 c. $F_1$ Plant
d. Hybridity confirmation using SSR marker and *cry1Ac* marker

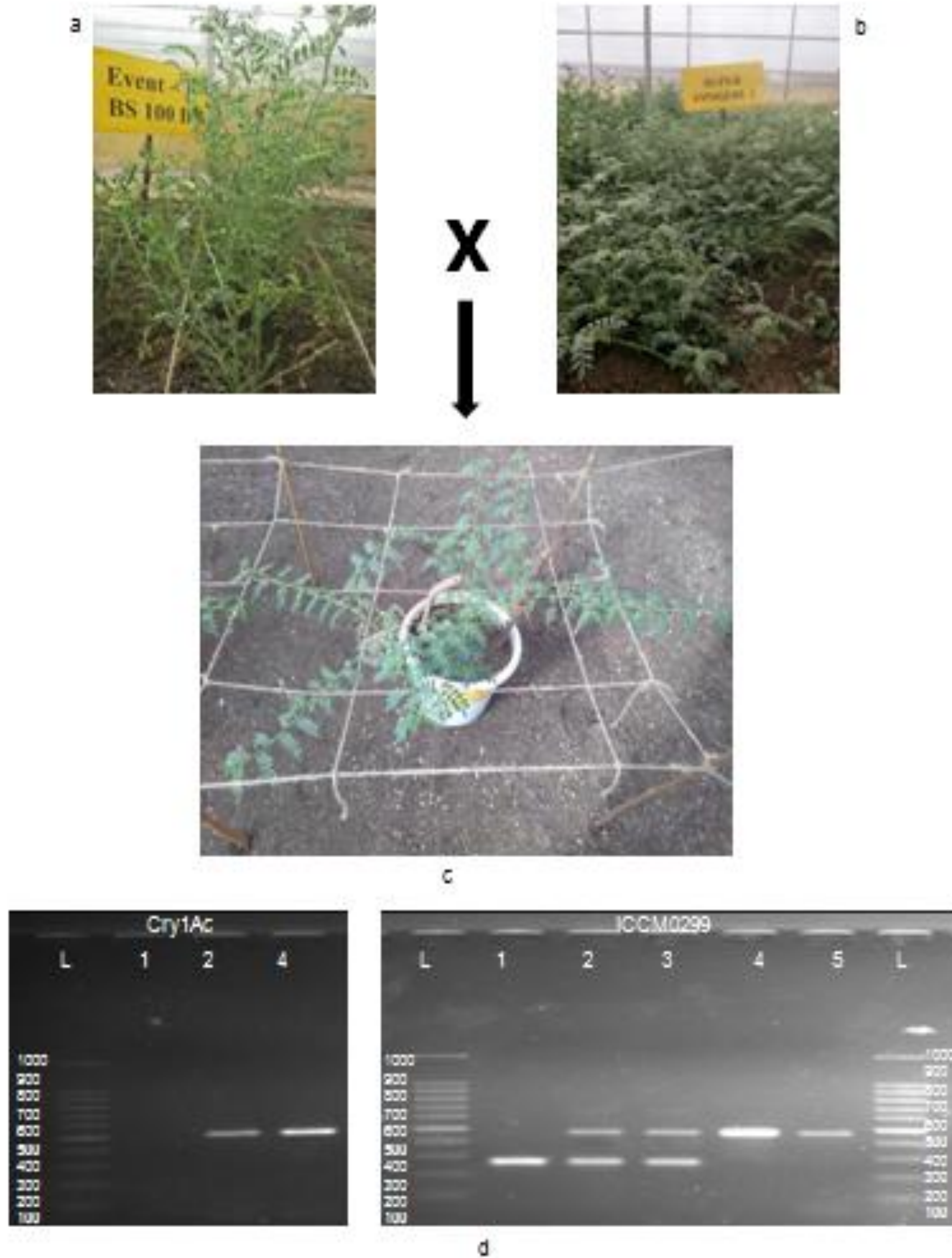


**Fig.2** *cry1Ac* gene segregation in $F_2$
a Genotyping of 1-21 $F_2$ plants for *cry1Ac* gene
b Genotyping of 22-39 $F_2$ plants for *cry1Ac* gene

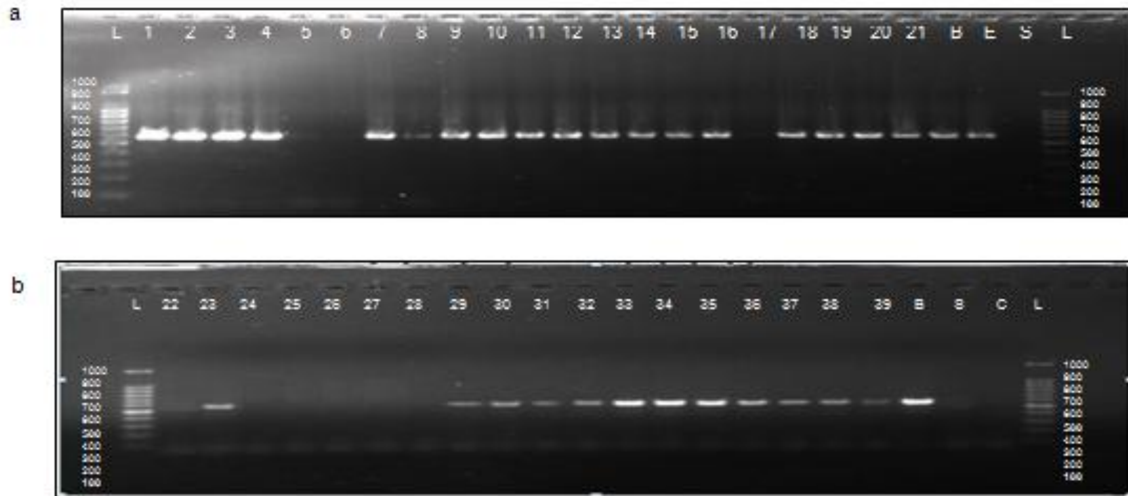



**Fig.3** Transcriptional and Translational level gene expression
*a. cry1Ac* gene expression confirmation at transcriptional level (RT PCR)
b. *Cry1Ac* protein estimation through quantitative ELISA

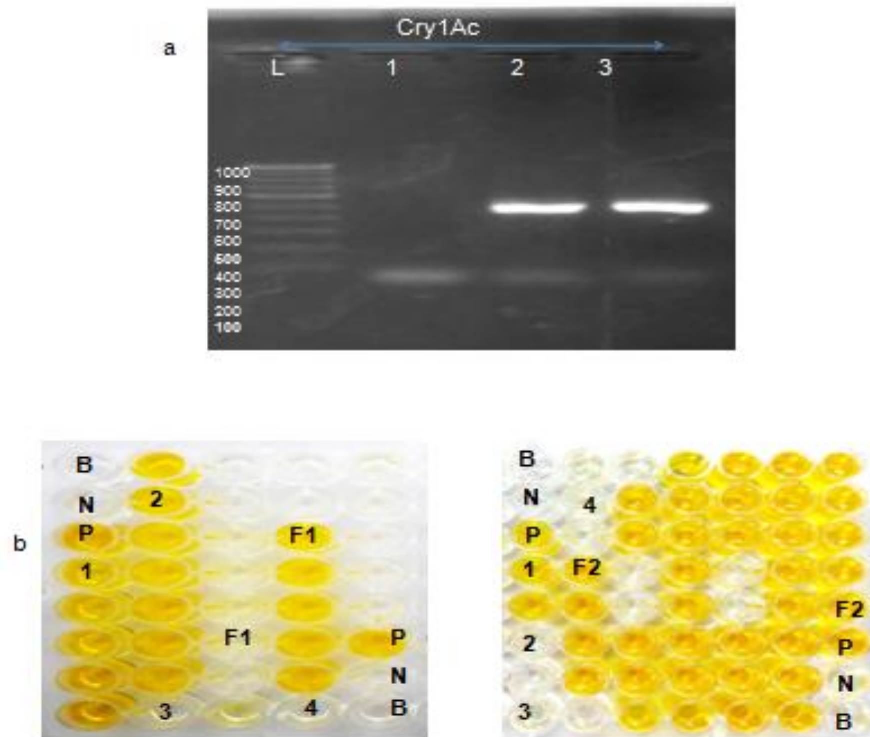


**Plate.1** Schematic representation of work flow

2017 SA-1 X BS 100B

Identified polymorphic markers

2018 $F_1$

Hybrid confirmed through polymorphic marker.
*cry1Ac*gene expression confirmed at transcriptional level through RT PCR and translational level through ELISA

2019 $F_2$

Studied inheritance pattern of *cry 1Ac* genes.
*cry 1Ac* gene expression confirmed at protein level through ELISA

**Plate.2** Identified three *Fusarium* wilt resistant markers

TS 82 TR 19 TA 96

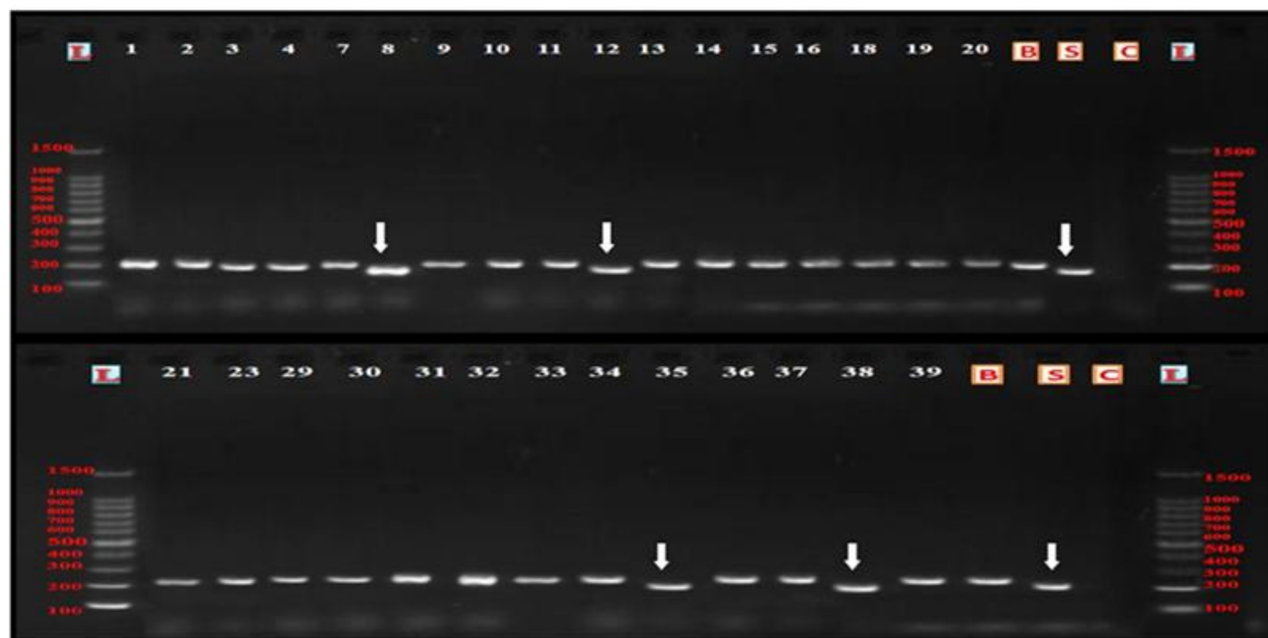
l- ladder (100bp), a-annigeri-1, s- super annigeri-1

**Plate.3** Identification of polymorphic wilt resistant marker

TS 82

b- bs 100b, e- bs 100e, c- bs 72c2, s- sa-1

**Plate.4** Identification of $F_2$ plants with both pod borer and wilt resistance




l- ladder (100bp), b- bs 100b (event-1), s- super annigeri-1, 1-39 *cry1a* positive f2 individuals,

c –control (without template)

In molecular breeding, identification of a greater number of background markers specific to recurrent (recipient) parent is useful in detection (background selection) of recurrent parent with its genome recovery at faster rate with more accuracy. Super Annigeri-1 and JG 74 improved lines by introgression of a *Fusarium* wilt resistant gene from WR 315 through marker-assisted backcrossing in chickpea were reported by Mannur *et al.,* (2019). This study used 38 polymorphic SSR markers for background selection and identified higher genome recovered plants with 90-95 per cent in $BC_2F_1$ and 90-97 per cent genome in $BC_3F_1$ in the cross Annigeri 1 × WR 315. In the case of cross JG74 × WR 315, 92-94 per cent recurrent parent genome was recovered in $BC_3F_1$ generation with the help of 42 polymorphic SSR markers. To identify markers specific to Super Annigeri-1 genetic background in the present study, 177 SSR markers evenly distributed in chickpea genome were screened for parental polymorphism on Super Annigeri-1 and BS100B and 13 (7.34%) and 91 (54.41%) markers showed polymorphism through agarose gel and automatic capillary electrophoresis, respectively. These polymorphic markers are useful to monitor background genome recovery of wilt resistant SA-1 in backcross progenies. The advantage of automatic capillary electrophoresis having ability to separate DNA fragments even at 3-5 bp, which is not possible in agarose gel electrophoresis, has also been realized in the present study. In the earlier study Mallikarjuna *et al.,* (2017) screened a total of 472 markers for parental polymorphism between the intra-specific crosses, ICCV 96029 × CDC Frontier, ICC 5810 × CDC Frontier, BGD 132 × CDC Frontier and ICC 16641 × CDC Frontier.

They identified only 6.86, 6.16, 5.04 and 4.06% polymorphism through agarose gel electrophoresis whereas, 21.18, 20.13, 19.07 and 19.70% through automatic capillary electrophoresis. Many earlier studies (Tekeoglu *et al.,* 2002; Udupa and Baum 2003; Tar'an *et al.,* 2007; Kottapalli *et al.,* 2009) reported of 30% to 70% polymorphism for SSR

markers between two parents of *Cicer arietinum* genotypes through automatic capillary electrophoresis.

In highly self-pollinated crops, artificial crossing is a difficult activity and in many times recovery of selfed seed even after crossing is encountered. Therefore, ensuring hybrid nature of the plant is essential before initiating further activities like crossing etc. In the absence of morphological markers to ensure hybrid nature of that plant can be confirmed with molecular markers. In our study, it was confirmed with two sets of markers, one with *cry1Ac* gene specific marker and another set with co-dominant SSR markers. Johnson *et al.,* (2019) used SSR 21 and SSR 22 markers to identify pure hybrid seedlings from the chickpea cross, Vaibhav x JG 97 and JG 11 x JG 97, JG 315 x ICCV 96029, and JG 14 x ICCV 96029. Similarly, Sharma *et al.,* (2018) confirmed the hybridity of Indian mustard (*Brassica juncea* L.) using SSR markers.

Understanding number of copies of gene integration in transgenic technology is important for handling the segregating populations during back crossing. Event of single copy of a gene is more desirable in backcross breeding provided it gives sufficient expression of a trait.

Therefore on analysis of segregation for *cry1Ac* gene in $F_2$ plants from Super Annigeri-1 × BS 100B (*cry1Ac* event) cross, it was confirmed a single gene inheritance, 3:1 genotypic ratio (PCR results) with significant Chi square test. Ripalda *et al.,* (2012) studied *cry1Ac* gene inheritance in backcross lines of crosses (Mara and EE-1) and (DLP and EE-1) and found significant Mendelian phenotypic ratios of 1: 1 and 3: 1 in $F_1$ and $F_2$ plants respectively, for a dominant single gene by demonstrating *cry1Ac* gene can stably inherit by means of back crossing. Transgenic event for any desirable trait in crops is commercialized either by cultivating that event itself as a variety or transferring that event to other genotypes known for wider adaptability.

In both the cases, it is always better understanding its stable integration by inheritance study on both integration and expression in $F_1/F_2$ generations. Sanyal *et al.,* (2005) produced stable transgenic plants with truncated *Bacillus thuringiensis (Bt) cry1Ac* gene driven by the *CaMV35S* promoter and identified stable integrated to plants by testing 3:1 segregation ratio of their progenies.

In the present study the confirmation of transcription of transgene in $F_1$ indicates stable integration of *cry1Ac* gene in donor parent. Mehrotra *et al.,* (2011) reported independent pattern of transgene expression due to random and complex integration in $T_0$ population whereas, in $T_1$ generation confirmed the stable integration and segregation of *cry1Ab* and *cry1Ac* genes.

In the present study even the expression of transgene at translation level is also confirmed through the qualitative ELISA tests in $F_1$ and $F_2$ generations and presence of around 20 µg per gram of fresh leaf tissue in donor, $F_1$ and $F_2$ plants strengthens the better utilization of the event as this level of Bt-protein is sufficient to kill pod borer. Sanyal *et al.,* (2005) also estimated the *Cry1Ac* protein accumulation which showed extractable protein with maximum range between 14.50 to 23.5 µg per gram of leaf tissue in $T_0$ and $T_1$ transgenic chickpea plants. Transformed chickpea plants expressing *Cry1Ac* protein above 10 µg per gram of leaf tissue soluble protein showed 80–85 per cent protection and high mortality (>80%) of insects while plants expressing between 5 and 10 µg per gram of leaf tissue resulted into early pupation, significant loss in weight (45–55%) and moderate mortality of insects. Similarly, Mehrotra *et al.,* (2011) observed expression of *Cry1Ac* toxins in transgenic chickpea in the range from 5 to 40 µg per gram of leaf tissue.

Although recipient parent for *cry1Ac* gene in the present study is wilt resistant, monitoring of the presence of wilt resistance genes while recovering recurrent parent genome in backcross breeding is essential. Varshney *et al.,* (2014a) reported six markers (GA-16, TAA-60, TA-194, TS-82, TA-110 and TR-19) linked to resistance to *Fusarium* wilt

races (*Foc 1* and *Foc 3*) and Mannur *et al.,* (2019) reported eight SSR markers (GA-16, TA-194, TA-27, TA-37, HIF05, CaM1158, TA-72 and TA-46) linked to resistance to *Fusarium wilt* race (*Foc* 4).

All these markers were further used for monitoring presence of *Fusarium* resistance alleles and estimating recurrent parent genome recovery. Three markers (TS-82, TR-19 and TA-96) showed polymorphism between isogenic lines (Annigeri- 1 and Super Annigeri- 1). Only TS 82 showed polymorphism between Super Annigeri-1 (wilt resistant) and BS 100B (unknown for wilt reaction), it will be used in further studies. Study will be continued to isolate transgressive seggregants through pedigree method for pod yield along with wilt and pod borer resistance using $F_2$ Plants. Backcross generated plants will be continued to backcross for another two to three cycles to derive perfect Super Annigeri-1 with pod borer and wilt resistance.

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