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1 Acknowledgments

This Small Research Activity (SRA) funded CSIRO to establish a multi-institutional collaboration between the Bangladesh Agricultural Research Institute (BARI), Assam Agricultural University and CSIRO with the aim of releasing insect resistant (IR) chickpea in Bangladesh. CSIRO appreciates ACIAR's support for this work.

The group also thanks the McKnight Foundation, Indo-Swiss Collaboration in Biotechnology and the Australia India Strategic Research Fund for financial support to generate transgenic IR chickpeas. The AAU team acknowledges funding received from the Indian Government's Department of Biotechnology and the Indian Council of Agricultural Research. We acknowledge support received from BARI, Bangladesh

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2 Executive summary

Chickpea is one of the important food legumes in Bangladesh and is a good source of protein which makes it a healthy alternative to meat for poor and middle-class people of south Asian countries. Chickpeas are now not widely grown in Bangladesh and the strong domestic demand for chickpea relies heavily on imports from Australia, Canada and India. The government of Bangladesh wishes to increase the production of pulses, including chickpea, to achieve food and nutritional security. In 2019, chickpea cultivation helped small and marginal farmers reap some benefits because of reduced water consumption, reduced fertilizer and other inputs. However, *Helicoverpa armigera* and bruchids remain major production constraints both in the field and during storage, respectively. So far, breeders have been unable to provide insect-resistant (IR) chickpea varieties due to the lack of germplasm with host resistance.

The possibility of preventing yield losses in chickpea using genetic engineering is attractive and has been successful in other legumes such as soybean and cowpea, based on insecticidal proteins from soil bacterium *Bacillus thuringiensis* (Bt). Expression of Bt insecticidal proteins (*cry1Ac* and *cry2Aa*) in chickpea has already been shown, in proof of concept experiments, to confer resistance to *Helicoverpa*. Similarly, the bean α -amylase inhibitor (α -AAI) gene has been shown to protect chickpea grain from bruchid damage by 98% in the laboratory. Based on our survey of major chickpea growing areas, Bangladeshi farmers are aware of the benefits of genetically modified (GM) food crops, largely due to deregulation of IR Bt-brinjal in 2016 and are likely to adopt a similar IR technology for chickpea pests if it was available. Therefore, IR chickpea lines have the potential to address insect pests of chickpea in Bangladesh; however, adequate resources are needed to achieve significant impact. Through this SRA we planned to evaluate selected IR chickpea lines in confined field conditions in Bangladesh. We performed detailed molecular characterization of IR chickpea lines and prepared a draft Biosafety Dossier to comply with regulatory guidelines of Bangladesh. The application to conduct confined field trials using selected chickpea lines was placed with the Bangladesh National Technical Committee on Crop Biotechnology (NTCCB) and we await approval.

We propose that pyramiding different Bt genes in a single background could provide a sustainable level of resistance against *Helicoverpa*. Also, introgression of the bruchid resistance trait into Bt chickpea lines would be an ideal combination to protect from insect damage both in the field and during storage. Therefore, we recommend developing a 5-year project to translate the research activities of this SRA by generating a pyramided IR chickpea for Bangladesh.

3 Introduction

Importance of IR chickpea for Bangladesh

The Bangladesh economy is largely dependent on agriculture, which contributes around 15% of GDP. Agriculture is a source of income for the majority of the population. Rice is the major crop, but wheat, jute, sugarcane, pulses, spices, tea and fruits are also grown in Bangladesh. Although grain legumes occupy only 5% of the total cropped area; they play an important role in sustaining the rain-fed Gangetic flood plain area of Bangladesh. Legumes grown in that area now include lentil, grass pea, chickpea, mung bean, pea and black gram.

Chickpea ranks as the third most important pulse consumed in Bangladesh. The chickpea grain is consumed whole, split or processed; however, the green leaves and immature pods are also consumed as winter vegetables if they are available. The mature grain is soaked and boiled and used in many traditional Bangladeshi dishes. Chickpea flour is also used as a batter to produce fried food. To meet domestic demand, Bangladesh imported 205,000 tonnes of the chickpea worth of USD 150 million in 2017.

Chickpea is a winter crop in Bangladesh, mostly grown in the western (in Jessore, Jhenaidah, Magura, Faridpur, and Rajbari) and north-western regions (Barind tract). The desi chickpea is the predominantly cultivated type and only 5000 tonnes were produced in 2018 (FAOSTAT, 2018).

The 8th Five Year Plan (2021-2026) for Bangladesh agriculture division supports crop diversification to ensure food and nutritional security. Legumes were nominated to be part of crop diversification for sustainable farming to improve production and increase farm income. In 2019, chickpea cultivation in Rajshahi and the Barind Tract only resulted in the production of 1869 tonnes of chickpea from 1563 ha. Nevertheless, the farmers reaped the benefit of growing chickpea because of lower water consumption, as well as reduced fertilizer and other inputs (<https://www.bssnews.net/?p=211131>).

Although chickpea is a popular food legume in Bangladesh, there are many production constraints responsible for low yield. *Helicoverpa armigera* is a significant insect pest of chickpea (Chaudhary and Chaudhary, 1975, Chaudhury and Sharma, 1982) causing more than 30-40% damage to pods and under certain conditions the losses can be as high as 70-80% (Rahman, 1990). The low efficacy of microbial formulations, such as *H. armigera* nuclear polyhedrosis virus (HaNPV) and *Bacillus thuringiensis* and crop husbandry practices led to more reliance on chemical insecticides to manage this pest in the field (Gujar et al. 2000). One of the most cost-effective and environmentally sustainable methods would be to release GM chickpea expressing the *B. thuringiensis* genes.

Genetic engineering to develop insect resistant (IR) legumes such as soybean and cowpea varieties with complete resistance to pod borers was successful by using insecticidal genes of *B. thuringiensis*. These legumes are now approved for commercial cultivation and consumption. The Bt genes encoding proteinaceous endotoxins are already used as bio-pesticides in formulations containing live bacteria to protect food crops from damage by pod borer and other caterpillars. Two major Bt proteins, Cry1Ac and Cry2Aa, are known to be very toxic to *H. armigera*. The different modes of action of these toxins is dependent on different specific receptors in the pod borer midgut, making them unique and selective. The specificity of these proteins makes them ideal candidates for gene pyramiding through genetic engineering and both the *cry1Ac* and *cry2Aa* genes were used here for the generation of Bt-chickpeas.

Bruchids are economically important pests of stored pulses (Clemente and Cahoon 2004; Sharma 2001). Both *Callosobruchus maculatus* and *C. chinensis* cause significant damage (up to 30% loss) to chickpea grain during storage. The most common method of protection of chickpeas in storage is dusting seed with chemical insecticides. Interestingly, amongst the legumes, *Phaseolus vulgaris* is resistant to infestation by certain bruchids due to the presence of a gene encoding the α -amylase inhibitor 1 ($\alpha A11$) protein. When this gene was isolated from *P. vulgaris* and expressed in other pulses, including peas (Shade et al. 1994; Schroeder et al. 1995) and azuki beans (Ishimoto et al. 1996), the grains were resistant to damage by *Callosobruchus* spp in the laboratory

IR chickpea varieties containing both *Bt* and *$\alpha A11$* genes could therefore help the management of insect pests in a sustainable manner and improve chickpea production in Bangladesh.

4 Objectives

The availability of IR chickpea in Bangladesh would enhance sustainable crop diversification. The IR chickpea would be cost-effective for smallholder farmers because it could be grown with fewer chemical inputs. It could also help improve farm income as appears to be the case with Bt- Brinjal.

The government of Bangladesh has shown an interest in chickpea production to meet domestic demand. This led to the recent release of BARI Chhola-5, BARI Chhola-10 and BARI Chola-11, which together are heat tolerant, Botrytis Grey Mould resistant, and higher yielding, chickpea varieties.

One of the research priorities of ACIAR in Bangladesh is improving smallholders' livelihoods and human nutrition through diversification into legumes, such as mung bean and chickpea. Therefore, we proposed to release the IR chickpea in Bangladesh through this SRA.

The SRA had the following objectives:

- Identify traits required for chickpea to be an attractive crop for Bangladesh farmers.
- Establish the importance of the insect pest constraints for chickpea production in Bangladesh.
- Import insect-protected transgenic chickpea lines into Bangladesh, in compliance with Bangladesh regulations.
- Determine the response of the lines to insects, under confined trial conditions.
- Identify and validate lines suited for the future development of varieties that could be deployed in Bangladesh farmers' fields.
- Depending on previous objectives being achieved: Submit to ACIAR a large research proposal with the aim of developing locally adapted high yielding pod borer and bruchid-resistant chickpea cultivars.

5 Establish the importance of insect pest constraints for chickpea in Bangladesh

In 2017, scientists from ACIAR, the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Assam Agricultural University (AAU) and BARI visited several farmer groups in Bangladesh to identify production constraints of chickpea. Again in 2018, BARI scientists visited different chickpea growing areas and held farmer group discussions. Although several research publications from 2001-2008 indicated that *Helicoverpa* was a major pest of chickpea in Bangladesh (Zahid et al. 2008), the lack of recent data led to this survey. The survey confirmed the need for IR chickpea and this was recognized by BARI breeders and stakeholders alike.

The survey showed that Bangladeshi farmers were aware of the benefits of Bt-technology because of the successful adoption of Bt-brinjal and suggested that they would consider adopting other GM food crops. A discussion with the scientists associated with the release of Bt-brinjal also helped to understand the GM regulatory framework in Bangladesh.

Based on the results of the survey and extensive discussion with BARI colleagues, we initiated the regulatory approval process in Bangladesh for the deregulation of pod borer and bruchid resistant chickpea lines generated by an earlier CSIRO/AAU collaboration. We have collated information required for the Biosafety Dossier. A detailed molecular analysis was carried out on the existing IR chickpea lines, as briefly described below, so as to comply with NTCCB regulations.

6 IR chickpea in compliance with Bangladesh Regulations

Pod borer resistant chickpea

CSIRO and AAU generated Bt chickpea lines resistant to either pod borers or bruchids using *Agrobacterium*-mediated genetic transformation. Codon-optimized Bt genes encoding Cry2Aa and Cry1Ac were introduced into chickpea (Acharjee et al. 2010; Hazarika et al. 2019). It was known that the neonate larvae are voracious consumers of green leaves of the chickpea; therefore, an *Arabidopsis* rubisco small subunit gene promoter (*AtSSU*) was used to regulate the expression of the genes. Over 140 transgenic lines were generated, and lines expressing high levels of the Bt proteins in leaves and stem as well as in the reproductive organs were selected (See Table 1 and Appendix Table 1).

Southern blot hybridization showed the presence of a single copy of the *cry2Aa* and *1Ac* genes in selected lines (Acharjee et al. 2010; Hazarika et al. 2019; Appendix Figure 1). Segregation analyses of the T₁ progeny of selected lines showed that the genes in these lines behaved as a single dominant locus (Acharjee et al. 2010; Hazarika et al. 2019; Appendix Table 1). Insect bioassays on selected lines showed complete protection against *H. armigera* larvae in the 2Aa lines (Acharjee et al., 2010) and only 1.4% pod damage in the case of the 1Ac lines (Hazarika, 2019). The phenotype of the 1Ac plants was indistinguishable from the non-transgenics but the phenotypes of the 2Aa lines were less vigorous than the parent line. We propose that the 1Ac lines could be considered for inter-crossing to generate elite pyramided chickpea lines with enhanced protection against insect pests in the field. However, we have reservations about using the existing 2Aa lines for crossing and, as part of this SRA, new lines with 2Aa are being produced before proceeding with these crosses. Cry 2Aa lines will not be considered further here until new 2Aa lines are available (see later).

Bruchid resistant chickpea

A bean (*Phaseolus vulgaris*) gene encoding the $\alpha A11$ gene was used to provide resistance to bruchids in chickpea seeds (Sarmah et al. 2004) via *Agrobacterium*-mediated gene transfer. Lines accumulating $\alpha A11$ to about 4% of seed protein

strongly (over 98%) inhibited the development and emergence of *C. chinensis* and *C. maculatus* in insect bioassays.

The transgenic chickpea lines expressing *Cry1Ac* and *α A11* genes appeared to be suitable for crossing and pyramiding with elite Bangladesh chickpea varieties.

7 Detailed molecular analyses of selected IR chickpea lines for Bangladesh

We selected two pod borer resistant 1Ac lines, 100B and 100E and two bruchid resistant lines, 39C and 40D, based on their low transgene copy number, high levels of resistance and normal phenotypes. Because the lines were generated by *Agrobacterium*-mediated transformation, the integration of the transgene was random. Therefore, a detailed molecular characterization of IR chickpea lines was performed to quantify the level of expression of transgenes in various organs, determine the transgene integration pattern, the possible integration of plasmid DNA sequences in the chickpea genome, and the site of integration in the chickpea genome.

Selected Bt and α A1 chickpea lines were used to measure the concentrations of transgenic proteins in various organs and to characterize the nucleotide sequence of the entire inserted T-DNA including the 5' and 3' flanking sequences of the chickpea genome.

j) Concentration of the Cry and α A1 proteins in various chickpea organs

The concentration of the transgenic proteins Cry1Ac and neomycin phosphotransferase II (Npt II) was determined by Enzyme-Linked Immunosorbent Assay (ELISA). Total proteins were isolated from the leaf, flower, stem, pod, green cotyledon and dry seeds of homozygous Cry1Ac and α A1 lines in a PBST (Phosphate Buffer Saline with 0.07% Tween) buffer.

The highest level of Cry1Ac protein was detected in the flowers (100B; 94.5 μ g/ g FW, and 100E; 126 μ g/ g FW) followed by leaves (100B; 88 μ g/ g FW, and 100E; 120 μ g/ g FW). Cry1Ac protein was also found in immature pods and stems, while very low levels of Cry1Ac protein were detected in the roots and dry seeds (Table 1). High levels of α A1 were found in the seeds of lines 39C and 40D (Table 1) and were equivalent to the levels in bean seeds (Sarmah et al. 2004). The protein was not detected elsewhere in the plants as expected since a seed-specific gene promoter was used to control expression.

Table 1: The concentrations of Cry1Ac and α AI1 proteins in the various organs of chickpea lines, 100B, 100 E, 40D and 39C

Organ	Cry1Ac(ug/g FW)		α AI (ug/mg protein)	
	100B	100E	40D	39C
Leaf	88	120	0	0
Stem	23	35	0	0
Roots	0.22	0.54	0	0
Flower	94.5	126	0	0
Pod	23	15	0	0
Imm Cots	41	62	85	95
Dry Seed	1.1*	3.15*	35	40

*The concentration of Cry 1Ac in the dry seed was calculated as micrograms per gram dry weight.

ii) Concentration of Npt II in various organs

The average concentrations of the Npt II protein in the various organs of the transgenic lines were determined. The highest level of Npt II was detected in stems, ranging from 0.33 to 0.45 μ g/ g FW in the case of the Bt lines, and from 0.12 to 0.36 μ g/ g FW in the bruchid resistant lines. Npt II was not detected in pods, immature cotyledons or roots, but was detectable in dry seeds (Table 2).

Table 2: The concentration of Npt II in various organs of transgenic chickpea lines.

	NPT II (ug/g FW)			
	100B	100E	39C	40D
Leaf	0.329	0.302	0.241	0.316
Stem	0.413	0.459	0.332	0.359
Roots	0	0	0	0
Flower	0.368	0.15	0.264	0.115
Pod	0	0	0	0
Immature Cotyledons	0	0	0	0
Dry seeds*	0.25	0.28	0.262	0.261

*The concentration of Npt II in the dry seed was calculated as micrograms per gram dry weight

iii) Testing for the presence of plasmid backbone or junction DNAs in the IR chickpeas

Southern blotting of DNA from the transgenic lines was used to test for the integration of any plasmid backbone sequence, including the junction sequences between the two T-DNAs in pBK 203 (Appendix Fig. 2). The DNA from each line was digested with *EcoRI* followed by separation of fragments in a gel and transfer to a nylon membrane. No hybridization of the probes for backbone or junction was detected in either the Cry1Ac and α AI1 lines indicating lack of integration of backbone or junction sequences (Appendix Fig. 3 and 4). However, when genomic DNA of line 100B was subjected to whole genome sequencing, a small portion (300-800bp) of the backbone was detected. These data eliminate line 100B as a possible parent in breeding as the presence of vector backbone sequences in the genome will not be acceptable to the regulators. Further analysis of line 100E is in progress.

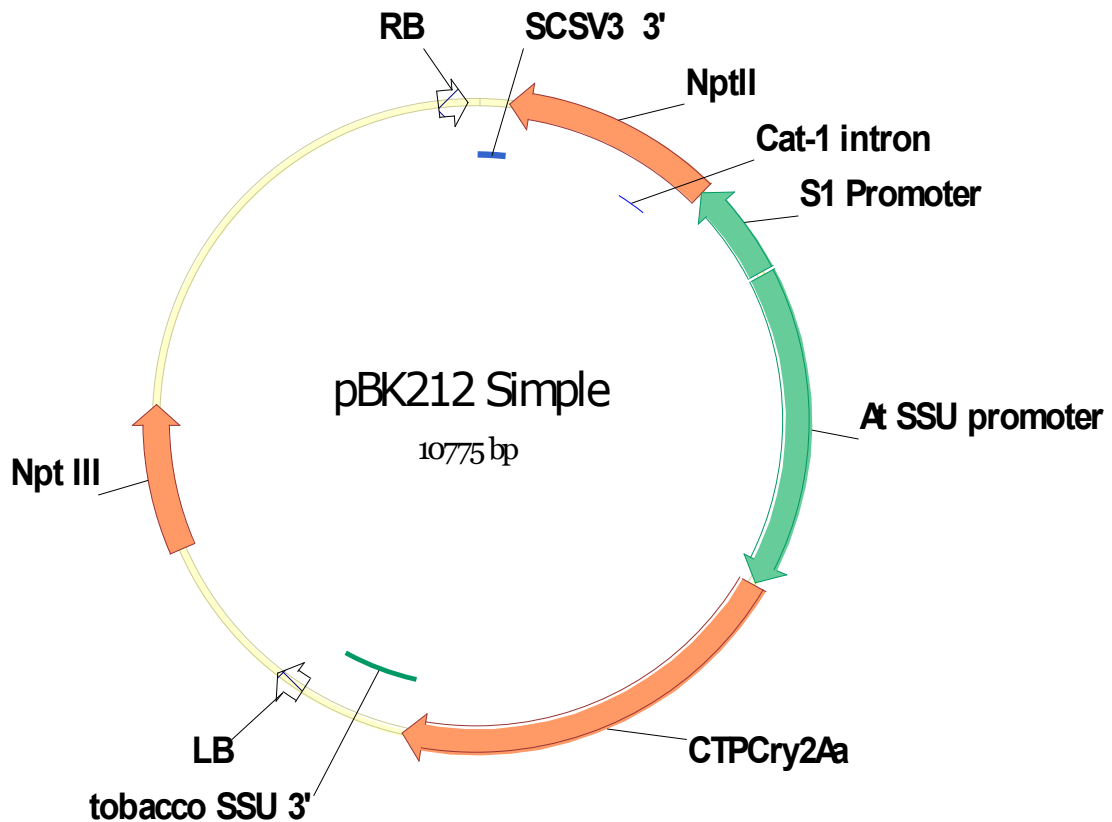
When genomic DNA of the $\alpha A11$ line, 40D, was subject to whole genome sequencing, it too, showed the presence of backbone sequence and possibly a second copy of the $\alpha A11$ gene. This line would not be suitable as a parent for breeding as it would not pass the regulatory requirements. The $\alpha A11$ line, 39C, is now being analysed by genomic sequencing

8 Generation of new Bt chickpea lines

To obtain regulatory approval of Bt chickpea in Bangladesh it is mandatory that the lines should be free of any vector backbone sequences. Our analyses through this SRA showed that the selected Bt chickpea lines have a small fragment of the vector backbone making them unacceptable for release. Therefore, we are generating more Bt lines and performing whole genome sequencing of Bt line, 100E and α AI1 line 39C, to test for vector backbone sequences. We are now performing genetic transformation of chickpea using a chloroplast targeted 2Aa gene construct (pBK212; Fig.1A) and a two Bt gene construct (pBK213; Fig.1B).

In both constructs, the 5' end of the 2Aa gene was fused to a chloroplast-targeting peptide (CTP) sequence, from the Arabidopsis Rubisco SSU 1A gene to target the 2Aa protein to the chloroplast with a view to reducing the phenotypic aberrations which we observed in our previous 2Aa lines.

A



B

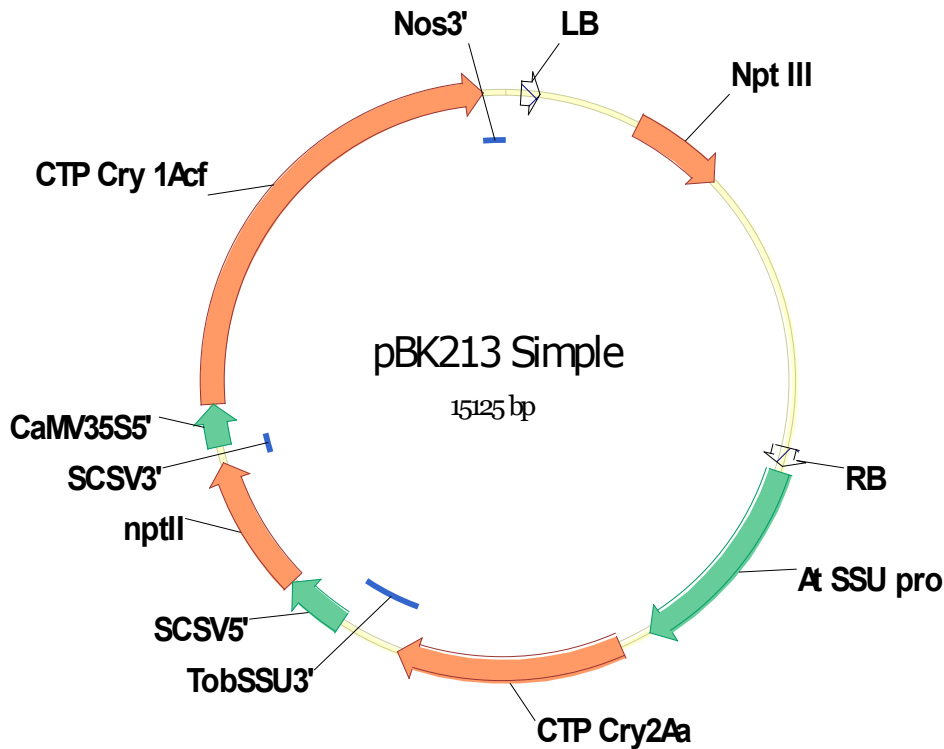


Fig 1. The Cry2Aa gene and the two Bt gene expression cassettes for chickpea transformation. A; In pBK212 the CTP represents the chloroplast-targeting peptide sequence from the Arabidopsis 1A Rubisco small subunit (SSU) gene. The Cry2Aa gene is controlled by Arabidopsis thaliana small subunit (At SSU) gene (ats A1) promoter (At SSU) and has a 3' region from *Nicotiana tabacum* small subunit (Tob SSU) in both pBK 212 and 213. B; In pBK 213 a full length Cry1Ac (Cry1Acf) gene was driven by Cauliflower Mosaic Virus 35S (CaMV35S) promoter and a nopaline synthase (NOS) 3'. The neomycin phosphotransferase II (Npt II) coding region was controlled by the subclover stunt virus segment 1 promoter (SCSV 5') and by subclover stunt virus segment 3' 3' region (SCSV3').

Using these two constructs we developed transgenic lines and have tested several so far. Using the two Bt gene construct, pBK213, we established 25 lines and so far, and tested 9 lines of which 6 were expressing both genes. Similarly, using the pBK212 construct we have transferred 16 lines to soil and found 2Aa expression in 3 lines (Table 3).

Table 3: Chickpea lines expressing either both Cry2Aa and Cry1Ac genes or Cry2Aa alone

Lines	Expression of Cry proteins (µg/g FW)	
	Cry1Ac	Cry2Aa
2 Bt genes Line		
19-92A	3.6	0
19-90C	5.2	1.7
19-85A	3.8	4.8
19-85H	1	1.5
19-86B	30	12.3
19-82A	1.4	1.7
Cry2Aa lines		
19-95D	-	14.2
19-95H	-	1.3
19-97G	-	1.2

9 Conclusions and recommendations

9.1 Conclusions

The purpose of this SRA was to prepare for the release IR chickpea resistant to pod borers and bruchids in Bangladesh. The SRA facilitated collaboration between BARI, AAU and CSIRO and helped establish the importance of IR chickpea for smallholder farmers in Bangladesh through a survey carried out by the team. The awareness of Bt-technology within the farming community of Bangladesh, due to adoption of Bt brinjal, helped the survey team immensely. The team concluded that release of IR chickpea would have a significant positive impact on farm income and the farm environment as well as the economy of Bangladesh. A detailed molecular characterization of existing IR chickpea lines was carried out to comply with the GM crop regulations in Bangladesh. However, our initially selected lines had some vector backbone sequences, therefore, we are producing and characterizing more lines. For example, we are generating new lines with two different versions of the Bt genes in order to generate pyramided IR chickpeas. The next step will be for BARI to select the preferred Bangladesh-adapted chickpea variety (or varieties), receive permission from the Bangladesh National Technical Committee on Crop Biotechnology, and initiate the backcrossing of Bt genes in the selected variety. The BARI team is well equipped to continue the work as they are already experienced in releasing Bt Brinjal varieties. BARI is also developing transgenic late blight-resistant potato varieties. BARI has some infrastructure needs and would require a small controlled environment facility (greenhouse) to screen and advance the IR transgenic chickpea lines and five confined field trial blocks for multilocation trials.

9.2 Recommendations

The positive economics of GM soybean (in the Americas), cowpea (in Africa) and brinjal (in Bangladesh) with lepidoteran insect resistance suggest that the release of IR chickpea could have a positive impact in Bangladesh. IR chickpea would be useful not only to reduce yield losses, but also help create an environmentally sustainable agriculture in Bangladesh. We selected existing IR chickpea lines with a single-Bt toxin for confined field trials in Bangladesh; however, we now propose the release of pyramided IR chickpea lines having multiple Bt genes against pod

borers together with a bruchid resistance trait. The pyramided IR chickpea with enhanced insect resistance management is expected to be preferred in the future. Therefore, if the government of Bangladesh identifies IR chickpea as a priority for the development of the country's pulses sector, we recommend that pyramided IR chickpea, as described above, be the focus of a long term (5-year) project, building on this SRA's results.

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10.2 Publications produced by the project

1. Hazarika, N, Acharjee S, Boruah R, Sarmah BK, TJV Higgins et al. (2019) Enhanced expression of Arabidopsis rubisco small subunit gene-promoter-regulated Cry1Ac gene in chickpea conferred complete resistance to *Helicoverpa armigera* J. Plant Biochem. Biotechnol.. <https://doi.org/10.1007/s13562-019-00531-1>.
2. Acharjee S and Higgins TJV (2019) Genetic engineering of grain legumes: Their potential for sustainable agriculture and food and nutritional security. In "Genetic Enhancement in Major Food Legumes". Editors: KB Saxena, RK Saxena & RK Varshney. Springer 2020

11 Appendix:

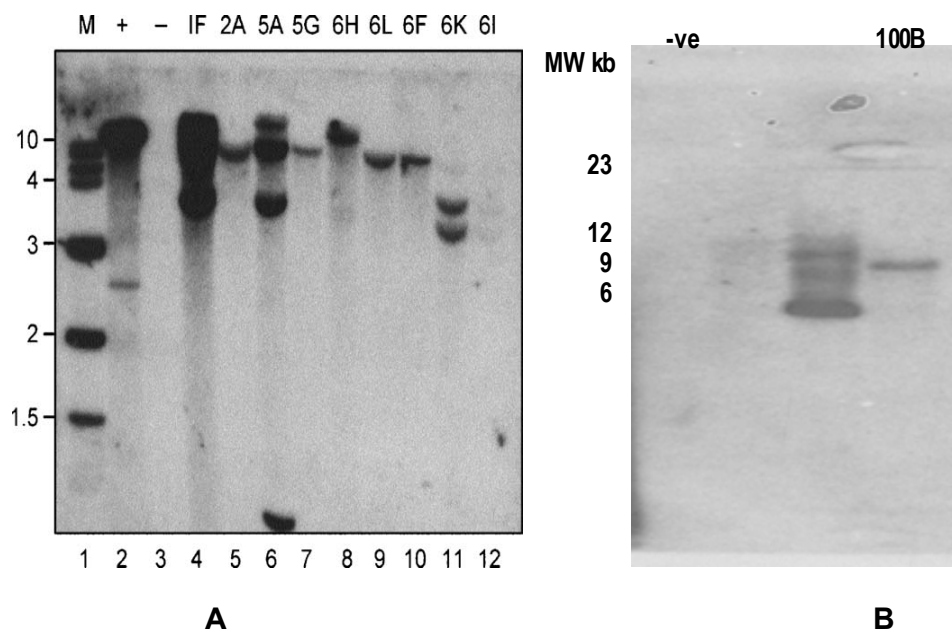


Fig 1: Southern blot analysis of transgenic Bt chickpea lines expressing either a Cry2Aa (A) or Cry1Ac (B) DNA was digested and the blot was probed with a PCR-amplified segment from within the *Arabidopsis thaliana* small subunit promoter (AraSSU). The numbers on the Y-axis refer to the sizes (in kilobase pairs) of the DNA markers (M).

Table 1: Molecular analyses of selected independent transgenic chickpea lines and segregation of the *Cry* genes in the T₁ generation

Lines	Segregation of <i>Cry1Ac</i> in T ₁ (+:-)	<i>Chi-square</i> χ^2	<i>P-value</i>
Cry1Ac lines			
100B	6:1	0.42	0.51
100E	7:1	0.66	0.41
Cry2Aa lines			
6H	19:4	0.71	0.4
6L	11:4	0.02	0.8

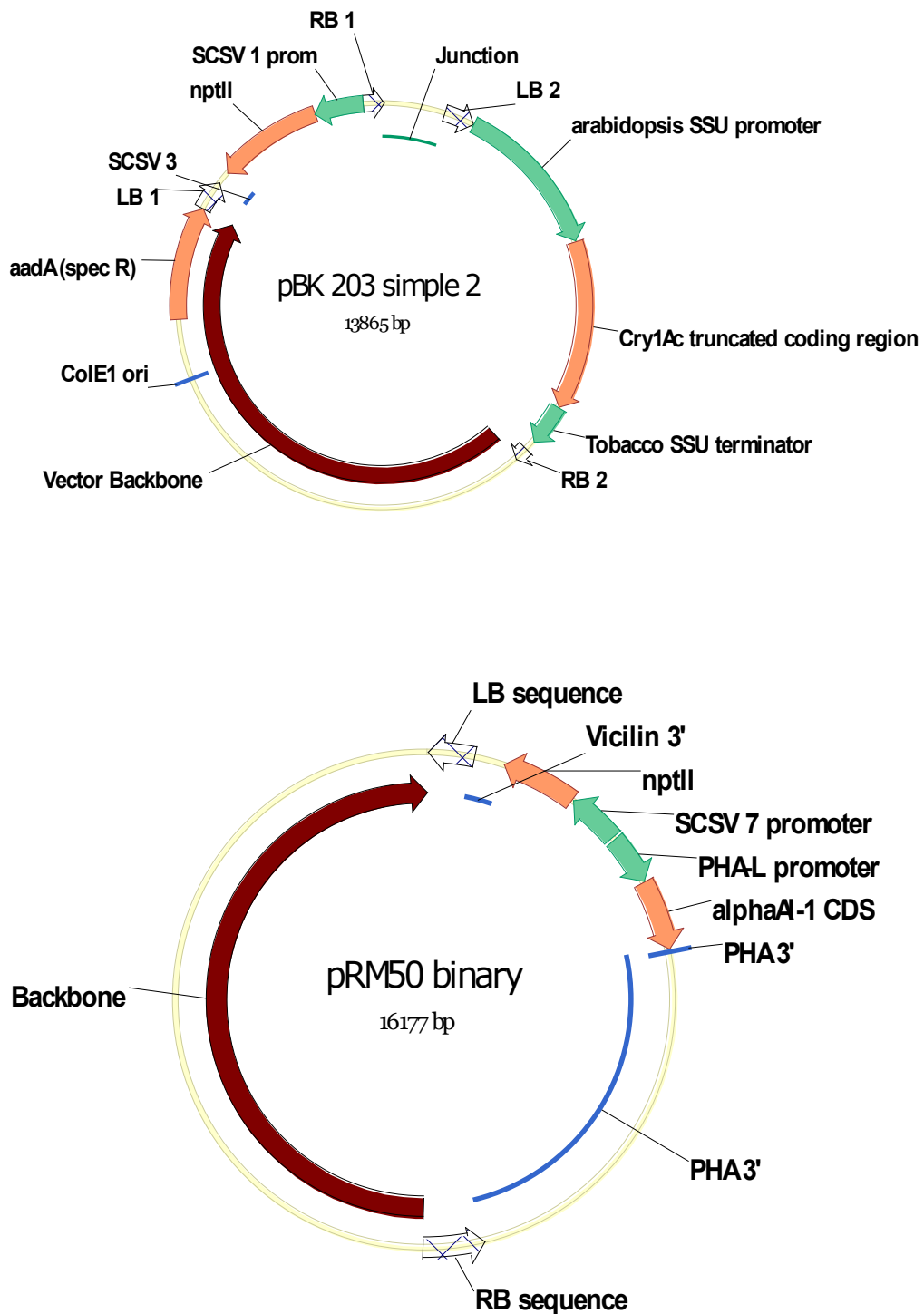


Figure 2. Map showing the vector backbones and junction probes used for the Southern blot hybridization for both Cry1Ac (pBK203) and α AI (pRM50) lines. The

region between the two T-DNAs (ie between RB 1 and LB 2) of the binary plasmid, pBK203 is the junction.

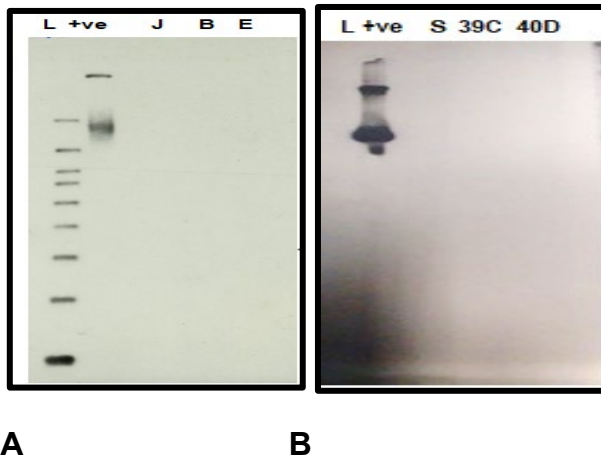


Figure 3. Southern blot to test for the integration of vector backbone sequence in the transgenic chickpea lines. (A) Genomic DNA isolated from the parental line, Jimbour (J) and transgenic lines, 100B (B) and 100E (E) was digested with EcoRI. PCR amplicons of the vector backbone of pBK 203 were used as probes to hybridize with the blot. (B) The genomic DNA isolated from the parental line, Semsem (S) and transgenic lines, 39C and 40D was digested with EcoRI. PCR amplicons of the vector backbone from pRM 50 were used as probes.

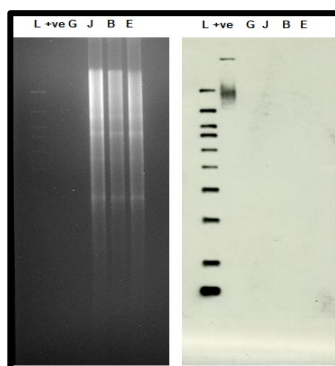


Figure 4: Southern blot of genomic DNA isolated from the parental line, Jimbour (J), and the transgenic lines, 100B (B) and 100E (E) probed for the presence of the junction region between RB 1 and LB 2 in pBK203. DNA was digested with EcoRI and a sequence corresponding to the junction was used as probe.