Developing Brassica napus lines with reduced pod shattering

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FINAL PROGRESS REPORT TO CANOLA COUNCIL
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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PAGE

1. SUMMARY:

We successfully completed the project "Developing *Brassica napus* lines with reduced pod shattering" and have achieved all the objectives. Three cDNAs (genes) involved in pod shattering in Arabidopsis were isolated, gene constructs (PS1, PS2 and PS3) in binary vectors were made, and a double haploid canola line (DH12075) was transformed with these constructs. Molecular and phenotypic analyses of the transformed plants were performed and several lines with reduced pod shattering were developed. Lines which showed reduced pod shattering (~50-80% less than control DH) and had normal pods were grown to successive generations to confirm their shattering resistance, for increasing homozygosity and for seed increase for field trial. After obtaining CFIA authorization, a 'confined research field trial' was completed. Despite many challenges we faced during the field trial, several lines with reduced pod shattering in the field (up to 30% less than control DH line) were identified.

After extensive searching for a silique-specific promoter (for use to drive pod shattering cDNAs), we isolated few potential candidates. One of these promoters (SPP, silique-preferred promoter) was selected for further use. The best performing Arabidopsis cDNA (IND1) was fused with this promoter to generate another gene construct (PS6), was inserted into canola, and transgenic lines with reduced pod shattering were developed.

Several potential genes involved in pod shattering resistance in *Brassica juncea* have been identified. We have successfully isolated cDNAs for five of these potential genes, prepared gene constructs (PS5, PS7, PS9, PS10 and PS12) driven by the SPP, and inserted them into canola. Several transgenic lines developed with these constructs (PS5 - PS12) showed reduced pod shattering in lab tests.

2. RECOMMENDATIONS:

1. Knowledge gained from this project resulted in identification of three *B. napus* homologues as potential targets for site specific mutation. Canola TILLING populations can be screened and target mutant lines can be used for breeding non-GMO canola cultivars with reduced pod shattering.
2. Transformed canola lines generated with *B. juncea* cDNAs should be further studied to confirm novel *B. juncea* gene/s responsible for pod shattering resistance in this species.

3. Lines with reduced pod shattering developed in this project can be made available to canola breeders; and the results of this project can be published so that breeders can use these germplasm and knowledge to develop pod shattering resistant canola lines.

3. PROJECT BACKGROUND:

Dispersal of seeds by 'pod shattering' mechanism is common in many plants like Brassica family (oilseed rape, Arabidopsis), soybean, sesame, etc. Although this is an important trait for plants' natural survival, it results in yield losses as much as 50% in cultivated crops (MacLeod 1981; Price et al. 1996). Current canola varieties are very susceptible to pod shattering and conventional breeding was not successful in developing resistant cultivars. Fortunately, recent advances in our knowledge in pod shattering in *Arabidopsis thaliana*, and discoveries of genes involved from this model Brassica plant, has made it possible to manipulate pod shattering in other Brassica plants. Several transcription factors have an important role in dehiscence process in Arabidopsis (Ferrandiz et al. 2000; Liljegren et al. 2000, 2004; Rajani and Sundaresan 2001; Mitsuda et al. 2005; Mitsuda and Ohme-Takagi 2008). Mutant Arabidopsis lines produce indehiscent siliques, and silencing of some of them (or over-expression) through genetic engineering gave Arabidopsis lines with non-shattering fruits. However, previous genetic engineering of *Brassica napus, B. juncea* and *A. thaliana* using pod shattering genes of Arabidopsis driven by strong CAMV35S promoter resulted in lines in which the pods did not open at all, and the plants showed various degree of sterility due to anther indehiscence (Vancanneyt et al. 2003, Mutsuda et al. 2005; Østergaard et al. 2006). These results demonstrate that to develop canola lines with reduced pod shattering, we need to reduce the strength of the used promoter and/or use a silique-specific promoter (that does not express in other tissues, especially anthers). Recently we have isolated a weaker (than CaMV35S) promoter from a forest vine *Macfadiana ungis-catii* (Macfadiana) and this promoter was used in this project.
We proposed to develop canola lines (*B. napus*) with reduced pod shattering by using three strategies: a) use a Macfadiana promoter to express several Arabidopsis transcription factors (NST1, IND1 and FUL) in canola; b) use a silique-specific promoter to express these genes; and c) isolate gene/s which are specific for pod shattering resistance in *B. juncea*, and express *B. juncea* gene/s in canola.

4. OBJECTIVES and DELIVERABLES OF THE PROJECT:

4.1: Key objectives were:

- Develop canola (*B. napus*) lines with reduced pod shattering as follows:
    1. Isolate/collect cDNAs (genes) for transcription factors (NST1, IND1 and FUL) from Arabidopsis.
    2. Prepare gene constructs with these cDNAs driven by a promoter from Macfadiana (a forest vine, old name is Doxantha, *Macfadiana ungis-catii*), and silique-specific promoter.
    3. Introduce these gene constructs into canola genome through *Agrobacterium* mediated transformation.
    4. Molecular analysis of transformed plants for transgene integration and expression.
    5. Analyze the transgenic canola plants for reduced pod shattering, and assess for petiole strength.
- Isolate cDNAs specific for pod shattering resistance from *B. juncea*, and use these gene/s to reduce pod shattering in *B. napus*.
- Field test the promising canola lines to confirm their reduced pod shattering in the field environment.
- Increase yield of canola through reduced pod shattering.
- Meet the increased demand of vegetable oils for bio-based industry.

4.2: Deliverables:

- Canola lines with reduced pod shattering.
- Several pod shattering gene constructs (for use in other crops as well, including camelina).
- Field trial data confirming greenhouse results of reduced pod shattering.
- Novel strategy for reducing yield loss from pod shattering.
- New scientific knowledge, publications, reports and patents.

5. RESEARCH PROGRESS:

5.1: Isolation of Arabidopsis cDNAs coding for transcription factors involved in pod shattering:

We successfully isolated three cDNAs coding for transcription factors (NST1, IND1, FUL) from developing siliques of Arabidopsis ecotype Columbia. The isolated cDNAs were cloned into PCR4-TOPO plasmid and sequenced to confirm their identity with the published GenBank sequences: AT2G46770.1 (NST1), AT4G00120 (IND1) and AT5G60910 (FUL). These cDNAs were then used for preparing constructs for canola transformation.

5.2: Isolation of cDNAs specific for pod shattering resistance from *B. juncea*:

Microarray analysis (performed at the Plant Biotechnology Institue, PBI, Saskatoon) of Arabidopsis *ful* mutant vs wild-type (WT), and *Brassica napus* vs *B. juncea* identified seven potential genes which might be responsible for pod shattering resistance in *B. juncea*. As a part of collaboration in this project with PBI, we received the sequence information of the Arabidopsis homologues of these genes (cDNAs; *At1* through *At7*). We have successfully isolated *B. juncea* homologue of *At1, At2, At3, At4* and *At5*, prepared five gene constructs (PS5, PS7, PS9, PS10 and PS12) with these *B. juncea* cDNAs (*Bj1, Bj2, Bj3, Bj4* and *Bj5*) as described.

5.3: Isolation of silique-specific promoter:

We have completed the search for, and isolating a silique-specific promoter to drive pod shattering genes/cDNA so that the effects of the transgene remain restricted to pods only and do not affect other tissues (especially anthers). After extensive investigation in public databases like Brassica Genomics Gateway, TAIR-The Arabidopsis Information Resource, BAR-Bio-Array Resources for Plant Functional Genomics, etc., we identified 25 Arabidopsis genes which might be good candidates for our search. We performed RT-PCR from RNA isolated from developing tissues of Arabidopsis ecotype Columbia to find expression profiles of these target genes. The expression pattern in different tissues did not really match with the information available in the public databases (Fig. 1). Therefore, we chose the two best possible candidates based on only public database information: AT4G22400.1 (P400) and AT3G21800.1 (P800). These two genes express only in developing fruits at either early or late stage. We isolated the promoter sequence of these two genes and sub-cloned upstream of the GUS reporter gene in the binary vector pGreen-GUS-NosT (Fig. 2). The constructs were confirmed by restriction digestion, introduced

into *Agrobacterium* strain GV3101 and re-confirmed by PCR analysis (Fig. 3). Canola line DH12075 was transformed with both constructs and the transformed plants were analyzed for GUS staining. The results showed that both P400 and P800 express in flower, seed and pod; but not in any vegetative tissues (root, leaf and stem).

We also made two more constructs with another potential silique-specific promoter, *AtALC* promoter and *BnALC* promoter. The promoter sequences were isolated and sub-cloned into a binary vector (Fig. 2). We introduced the construct in *Agrobacterium* and transformation of canola was done. Analysis of transgenic lines showed that these two promoters also express in flower, seed and pod; but not in any vegetative tissues (root, leaf and stem).

We successfully isolated a silique-specific (or at least silique-preferred, that mostly expresses in siliques) promoter (SPP) from Arabidopsis and prepared few gene constructs with this promoter. In parallel we grew next generation plants from this SPP-GUS construct for further analysis and confirmed its specificity. Analysis of second generation plants showed that this promoter mostly expresses in pods, with some expression in flower and seeds (Fig. 4).

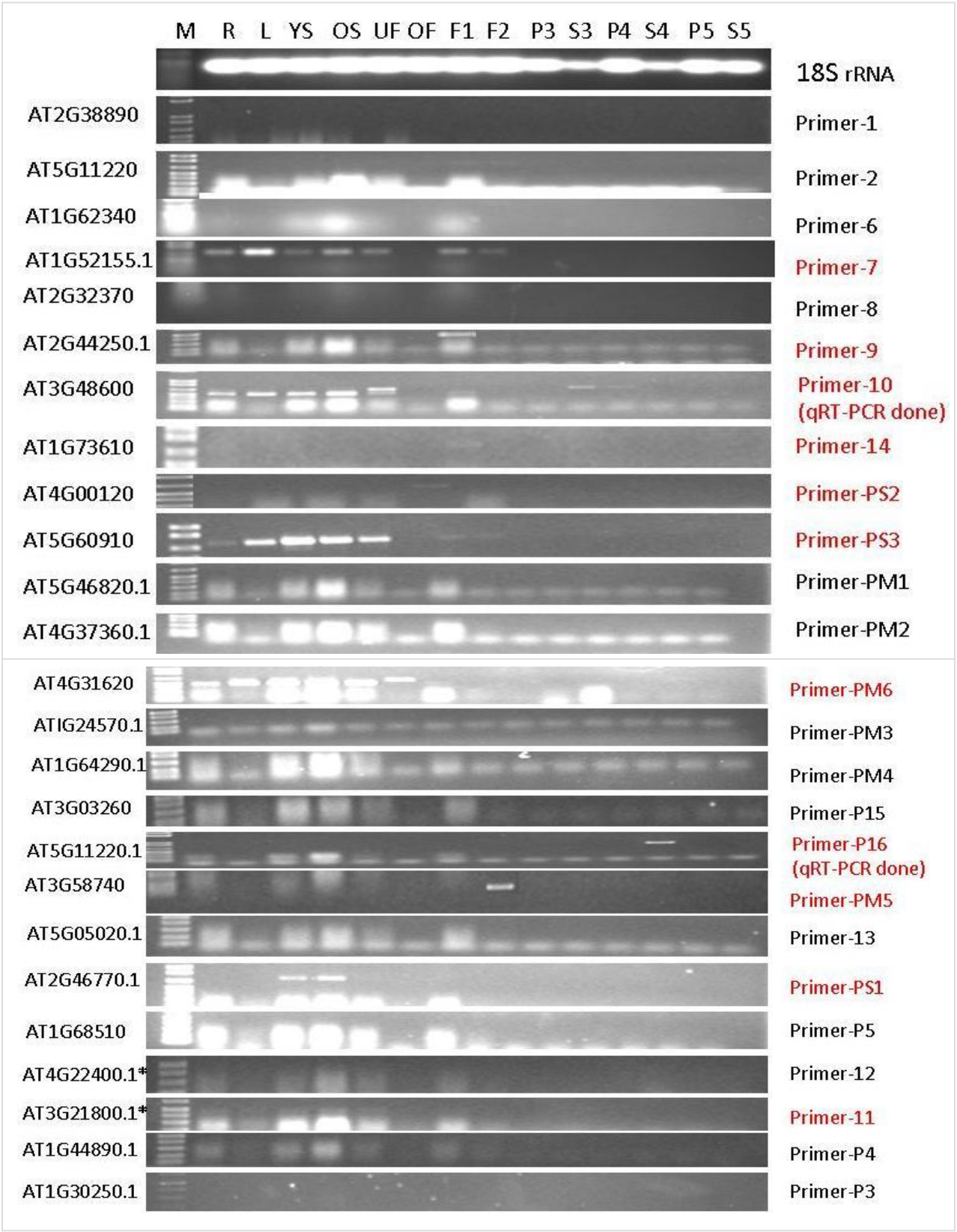


Figure 1: RT-PCR of various tissues from Arabidopsis for target genes. R= Root; L= Leaf; YS= Young Stem; OS= Old Stem; UF= Unopened Flower; OF= Opened Flower; F1= Fruit Stage 1; F2= Fruit stage 2; P3= Pod 3 (Fruit stage 3) (w/o seeds); S3= Seed 3 (Fruit stage 3) (seeds only); P4= Pod 4 (Fruit stage 4) (w/o seeds); S4= Seed 4 (Fruit stage 4) (seeds only); P5= Pod 5 (Fruit stage 5) (w/o seeds); S5= Seed 5 (Fruit stage 5) (seeds only)

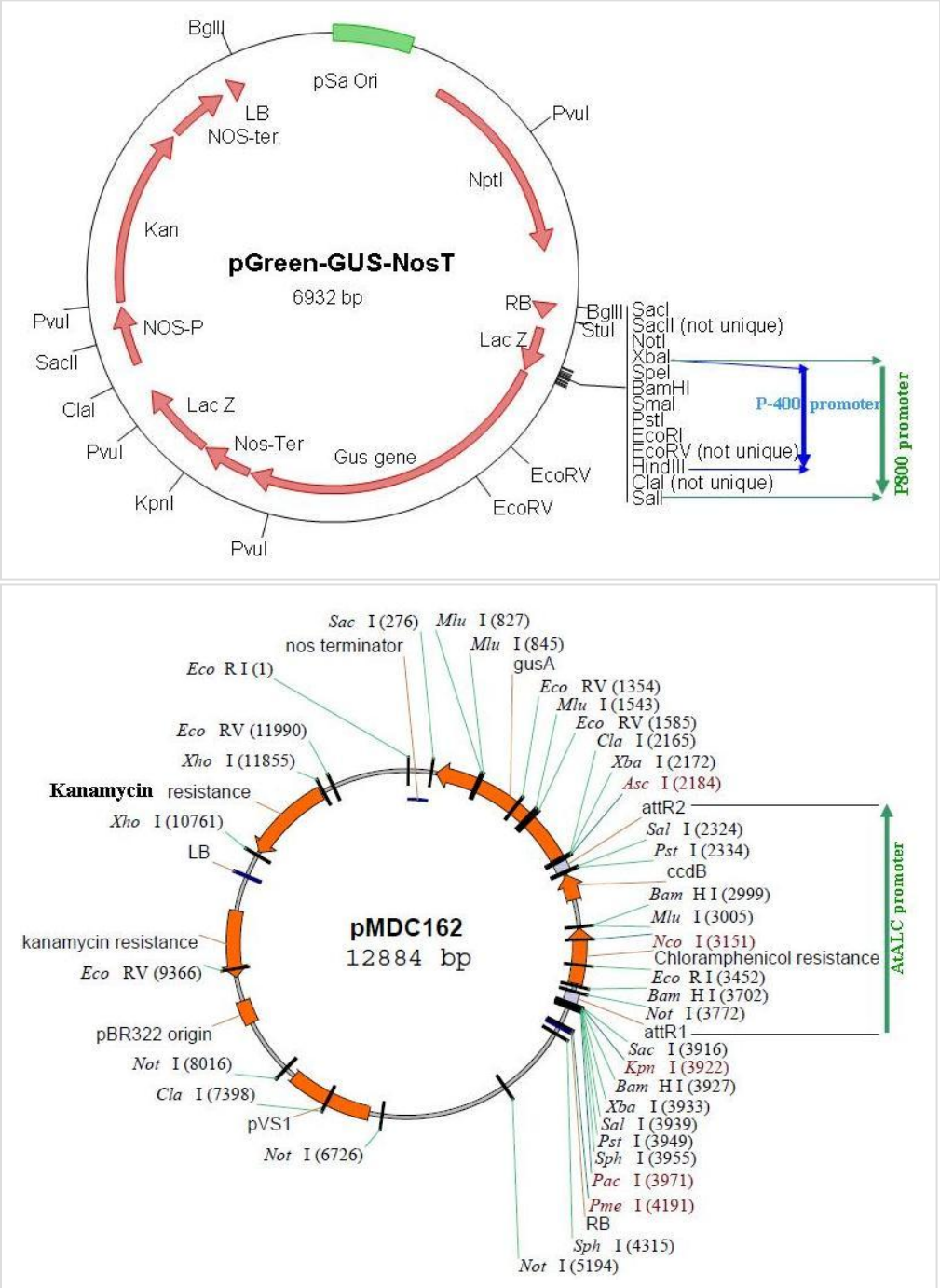


Figure 2: P400, P800 and AtALC promoter constructs for canola transformation

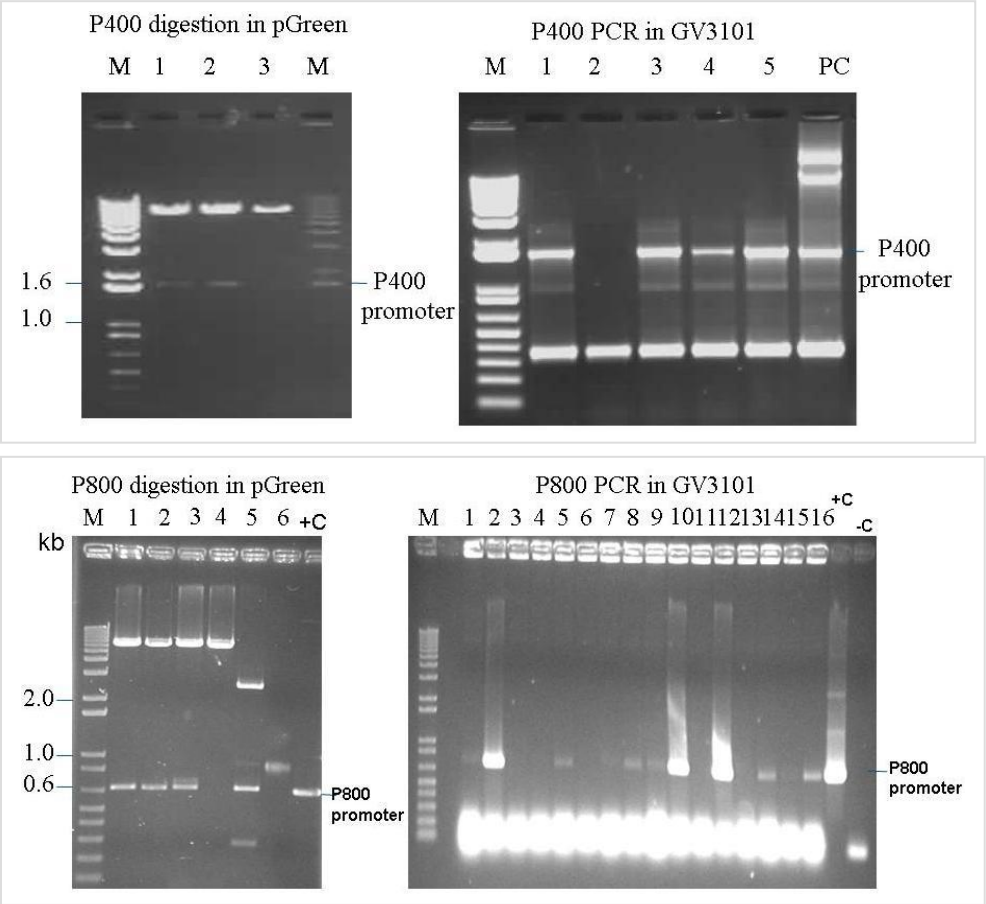


Figure 3: Restriction digestion and PCR confirmation of P400 and P800 constructs

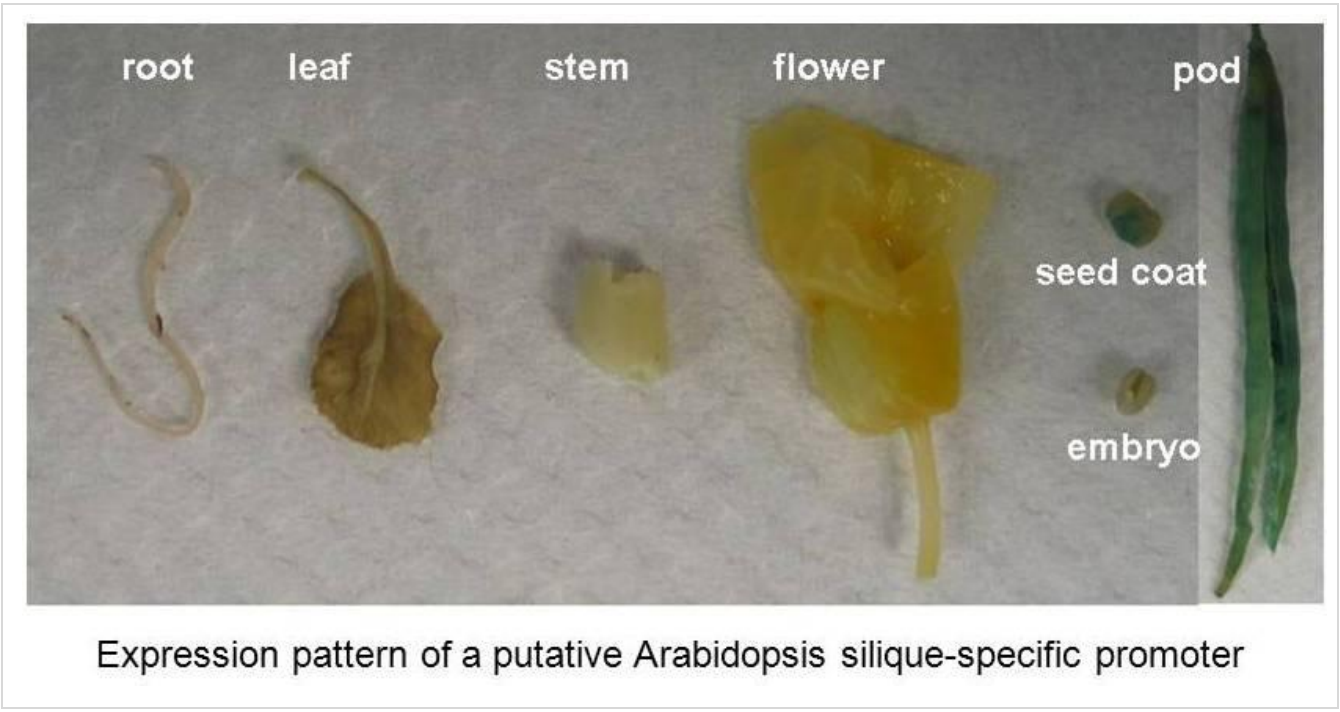


Figure 4: Expression pattern of a putative Arabidopsis silique-specific promoter

5.4: Preparing the gene constructs (PS1 to PS12):

PS1: NST1 gene (cDNA) sequence (AT2G46770.1) was amplified from cDNAs, synthesized from total RNA of developing Arabidopsis silique, using primers XbaINST1F (5' – GCTCTAGA***ATG***ATGTCAAAATCTATGAGCATATCAG – 3') and HindIIINST1R (5'-GCAAGCTT***TTA***TCCACTACCATTCGACACGT-3'). The forward primer had an XbaI site created immediately upstream of the ATG start codon and the reverse primer had a HindIII site created immediately downstream of the TAA stop codon. The PCR product was digested with HindIII and XbaI and ligated in antisense orientation in between the Macfadiana promoter and pea rbcS3'poly(A) terminator in pK3333 binary vector, which is a derivative of pKYLX71 (Schardl et al. 1987). The resultant vector was named PS1 (Fig. 5). Restriction digestion of PS1 confirmed the presence of NST1 cDNA (Fig 6A) and Macfadiana promoter (data not shown). Four PS1 clones were sequenced and clone #1 showed no mistake in DNA sequence. This clone was introduced into *Agrobacterium tumefaciens* strain GV3101 through freeze-thaw method (Chen et al. 1994). Seven *Agrobacterium* transformants were tested by PCR and all showed to carry the PS1 plasmid (Fig. 6B).

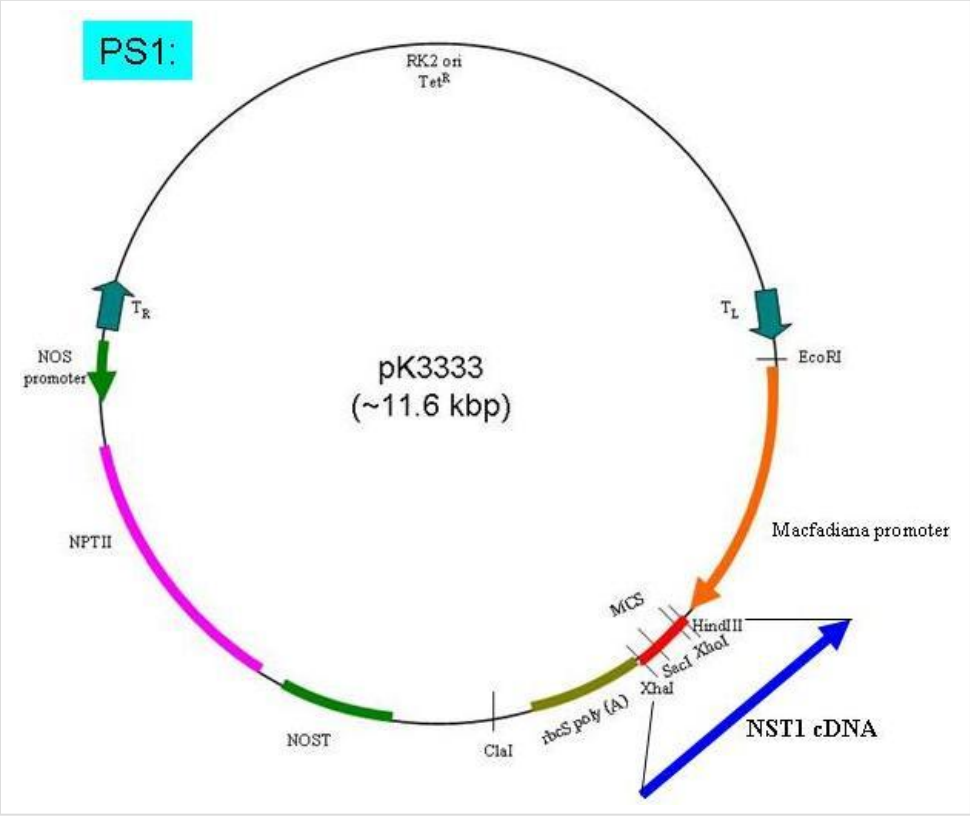


Figure 5: The gene construct PS1 was made in binary vector carrying NST1 cDNA in antisense orientation in between the Macfadiana promoter and pea rbcS3' poly(A) terminator

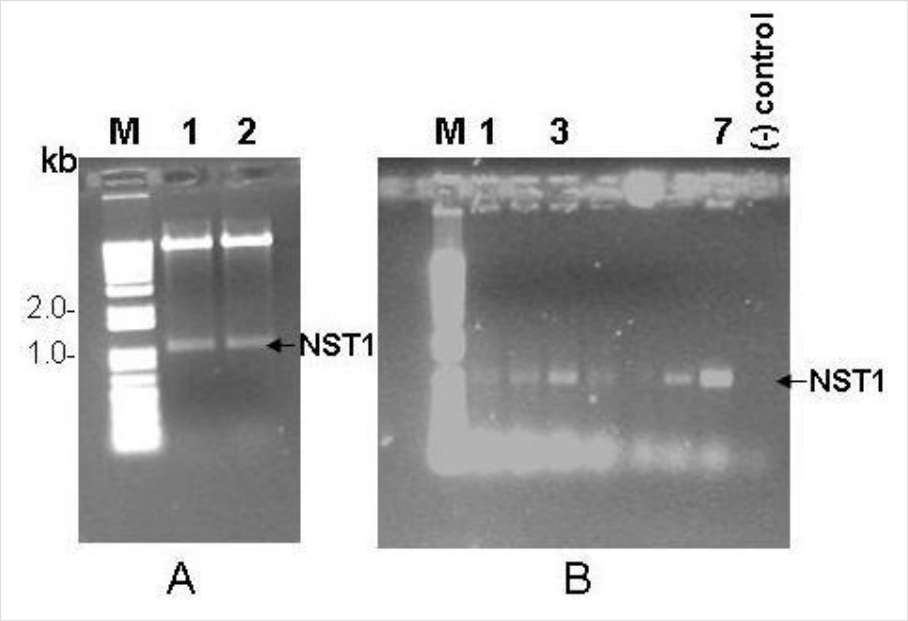


Figure 6: A, Restriction digestion of PS1 to show presence of NST1 cDNA in vector pK3333. B, PCR of transformed *Agrobacterium* to confirm presence of PS1 construct

PS2: IND1 gene sequence (AT4G00120) was amplified from cDNA, synthesized from mRNA of developing Arabidopsis silique, using primers XbaIINDF (5' – GCTCTAGA***ATG***GAAAATGGTATGTATAAAAAGAAAG – 3') and HindIIIINDR (5'-GCAAGCTT***TCA***GGGTTGGGAGTTGTG-3'). Like PS1, the forward primer had an XbaI site created immediately upstream of the ATG start codon and the reverse primer had a HindIII site created immediately downstream of the TAA stop codon. The PCR product was ligated to PCR4-TOPO plasmid. Four clones were sequenced and clone #1 and 3 had no mistakes. The IND1 fragment was taken out of clone #1 by HindIII and XbaI digestion and was ligated to pK3333 binary vector in antisense orientation. The resultant construct was named PS2 (Fig. 7), which was then restriction digested and sequenced to confirm its identity.

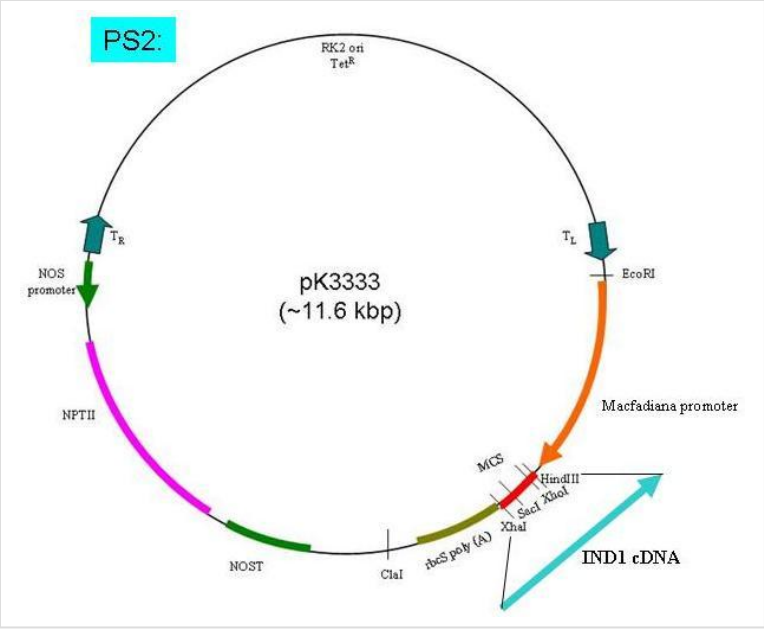


Figure 7: The gene construct PS2 was made in binary vector and carries IND1 cDNA in antisense orientation in between the Macfadiana promoter and pea rbcS3'poly(A) terminator

PS2 was introduced into *Agrobacterium* GV3101 and 12 putative transformed colonies were PCR tested for presence of the PS2 gene construct in them, out of which 3 clones (#3, 10 and 11) were positive (Fig. 8). Clone #10 was used for canola transformation.

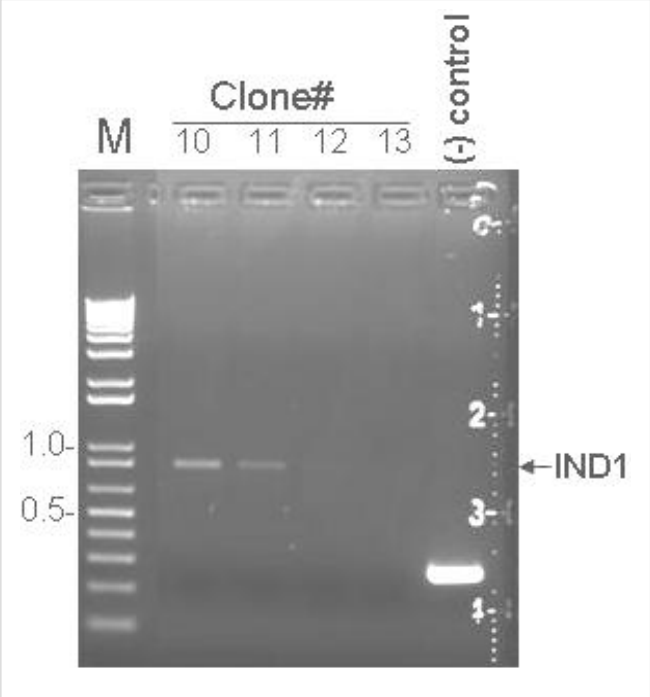


Figure 8: PCR of transformed *Agrobacterium* to test presence of PS2 construct. The PCR was performed with primers which give ~250 bp fragment in empty vector (-) control

PS3: FUL (AT5G60910) cDNA (gene) was amplified using primers: XhoIFULF: 5'-GCCTCGAGATGGGAAGAGGTAGGGTTCAG-3' and XhoIFULR: 5'-GCCTCGAGCTACTCGTTCGTAGTGGTAGGAC-3' from cDNAs synthesized from Arabidopsis mRNA. The FUL cDNA was ligated to PCR4 TOPO plasmid, two recombinant clones were sequenced; and clone #1 showed no mistake. In this clone, the start codon of FUL cDNA was towards the PstI site and the stop codon was towards the NotI site of pCR4-TOPO. The FUL cDNA was taken out from this clone with PstI and NotI and ligated to pGreen-napin-rbcS3 plasmid (Hellens et al 2000) to create "pGreen-napin-FUL-rbcS3".

Macfadiana promoter was amplified from pK3333 plasmid with primers: KpnDoxP-F: 5'-GTGG GGT ACC AGA GAC AAC ATA CTT C-3' and PstDoxP-R: 5'- GCC CTG CAG TTC TTC TTG TAC TTT GAG TGG-3'; digested with KpnI and PstI, and ligated to the above "pGreen-napin-FUL-rbcS3" to replace the napin promoter. Further analysis by restriction enzyme digestion showed that two clones (#11 &13) had FUL cDNA, Macfadiana promoter and rbcS3 terminator (Fig. 9A). Sequencing confirmed the identity of clone #11. This clone was

named PS3 (Fig. 10), was introduced into *Agrobacterium* GV3101 and confirmed with PCR analysis (Fig. 9B).

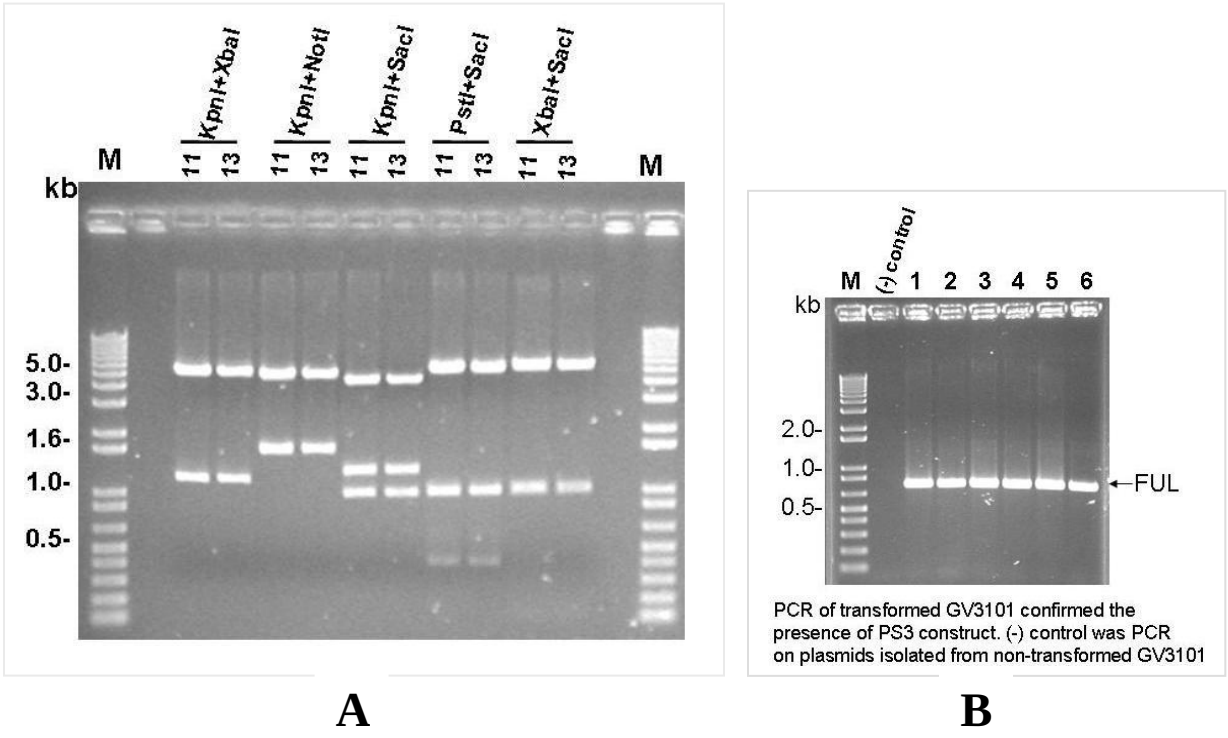


Figure 9: A, Restriction digestion of PS3 to confirm its identity. B, PCR of transformed *Agrobacterium* to confirm presence of PS3 construct.

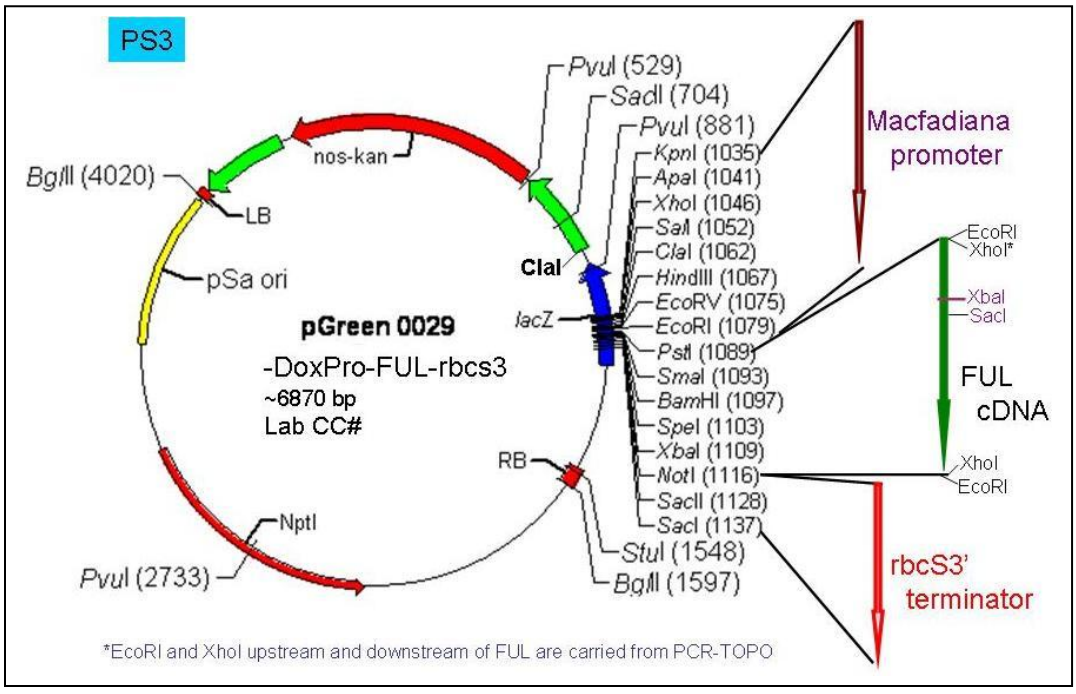


Figure 10: The gene construct PS3 was made in binary vector and carries FUL cDNA in sense orientation in between the Macfadiana promoter and pea rbcS3'poly(A) terminator

PS4 and PS5 (SPP:Bj1/pMDC100)**:** To prepare gene constructs PS5, 6, 7, 9, 10 and 12, we first made an intermediate plasmid, PS4. Our search resulted in the isolation of a few silique-preferred promoters (SPP) which mainly expresses in canola siliques at various developmental stages, with slight expression in other flower parts and seeds. They do not express in seedling, leaf, root, or stem. One of these SPP was amplified by primers SPPF-KpnI and SPPR-AscI, digested with KpnI and AscI, ligated to pMDC100 plasmids, and finally confirmed by PCR and restriction digestion. The resultant plasmid was named as PS4 (SPP-pMDC100).

The 459 base pair (bp) full length *Bj1* CDS (coding sequences), which is expected to be responsible for pod shattering resistance in *B. juncea*, was amplified by One-Step RT-PCR with gene specific primers using mRNA from two stages of developing *B. juncea* pods (Fig. 11A). The PCR products were ligated to TOPO TA pCR8 vector and colony PCR was used to identify correct clones (Fig 11B). Sequencing of these clones identified five clones (*Bj1-PCR-1,-5,-7,-8,-12*) with correct sequences and all were in appropriate orientation for recombination reaction (Gateway vector system, LR reaction). The best matching clone (*Bj1-PCR-1*), that had only one amino acid difference (I to V) from the published sequence in BB genome of *Brassica*, was used to recombine with above PS4 (SPP-pMDC100 vector carrying a silique-preferred promoter).

Colony PCR showed that all 7 colonies tested were correct (Fig. 11C) and two clones were further confirmed by restriction enzyme digestion (Fig. 11D). The construct was introduced into *Agrobacterium* GV3101 and checked by colony PCR (Fig. 11E). It was named PS5 (SPP:Bj1/pMDC100, Fig. 11F) and was used for canola transformation.

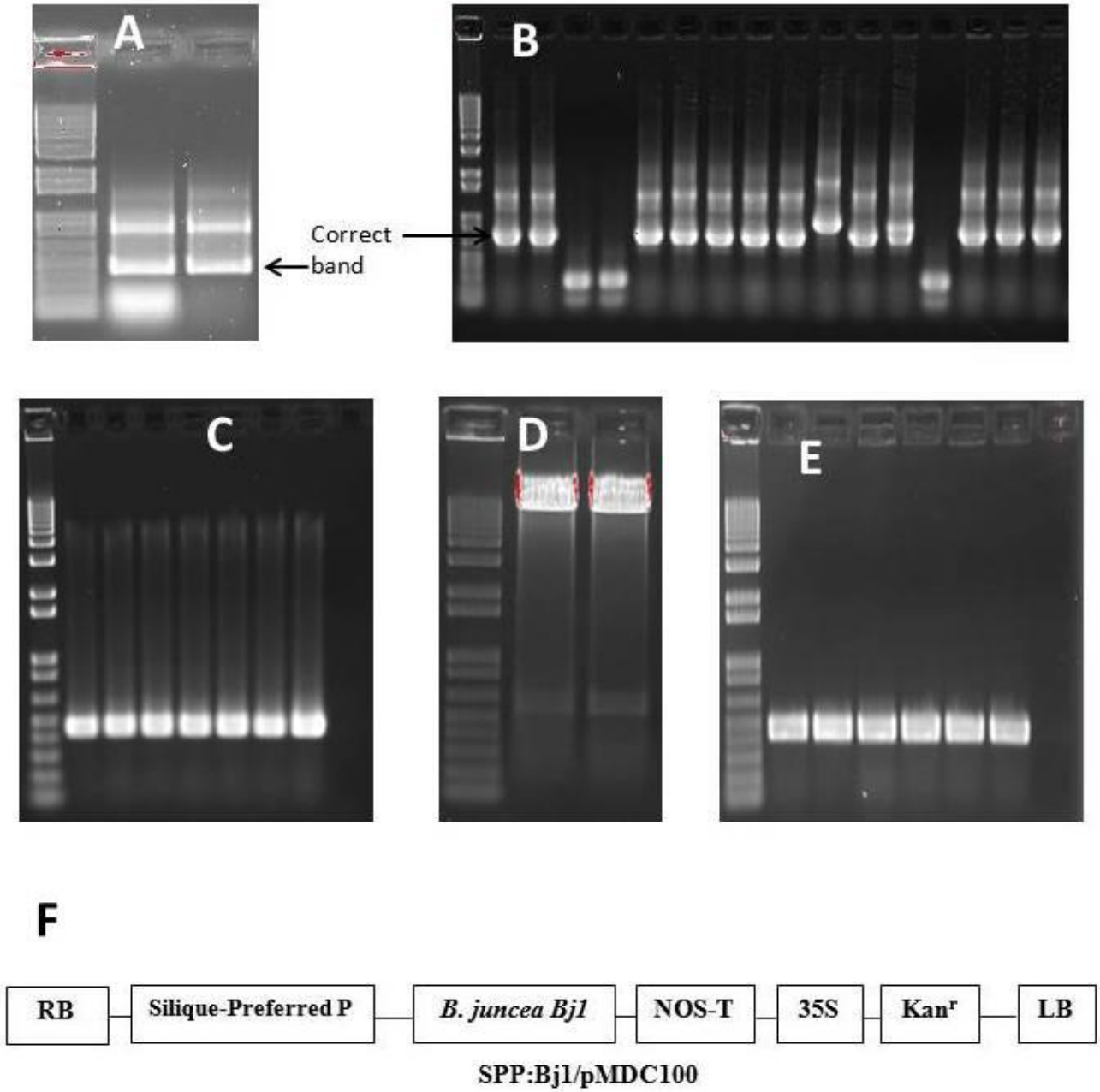


Figure 11: Preparing a gene construct (PS5) of *B. juncea* CDS *Bj1*, that is involved in pod shattering resistance, driven by a silique-preferred promoter

PS6 (Antisense IND1 cDNA in pMDC100 vector with SPP)**:** Arabidopsis IND1 cDNA (AT4G00120) was amplified from the plasmid IND-pCR4-TOPO (see PS2) using primers XbaIINDF (5' – GCTCTAGA***ATG***GAAAATGGTATGTATAAAAAGAAAG – 3') and HindIIIINDR (5'-GCAAGCTT***TCA***GGGTTGGGAGTTGTG-3'). The PCR product (Fig. 12A) was ligated to pCR8-TOPO-TA plasmid (Fig. 12B) and was sequenced to check identity and orientation. The plasmid from one clone that had the antisense orientation (IND-pCR8#1) was used for the Gateway LR cloning with the site specific recombination in pMDC100 vector

carrying the silique-preferred promoter (SPP) that we had isolated. After ligation, the recombinant plasmid was transformed to One shot OmniMAX 2T1 *E.coli* competent cells; and its identity and orientation was confirmed by colony PCR (Fig. 12C), restriction digestion (Fig. 12D) and sequencing. The resultant construct was introduced into Agrobacterium GV3101 by electroporation and the Agrobacterium colonies were tested by colony PCR (Fig. 12E) to confirm the presence of IND gene. The final construct was named PS6 (SPP:Anti-IND1/pMDC100 (Fig. 12F) and was used for canola transformation.

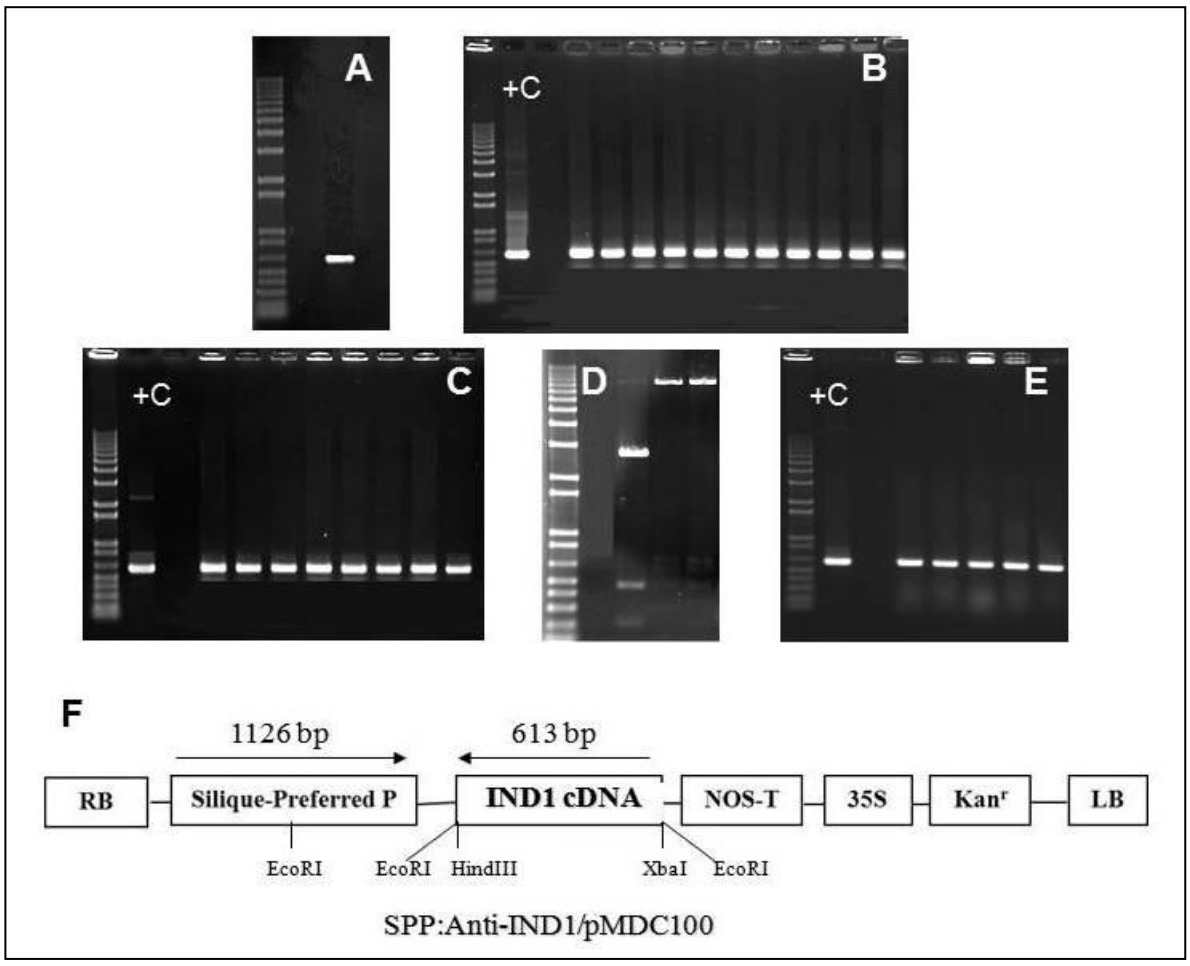


Figure 12: Preparing the gene construct (PS6) in binary vector pMDC100. It carries FUL cDNA in antisense orientation in between the silique preferrd promoter and NOS poly(A) terminator

PS7 (SPP:Bj2/pMDC100): The *B. juncea* homolog (*Bj2*) of Arabidopsis pod shattering related gene *At2* was amplified by One-Step RT-PCR with 3 sets of gene specific primers (Bj2F & Bj2R for PCR1 reaction; Bj2olCDSF & Bj2olCDSR for PCR2 reaction; and At-Bj2raCDSF & Bj2raCDSR for PCR3 reaction) and *B. juncea* RNAs. PCR1 and PCR2 gave desired results and each had two bands including target bands (Fig. 13A). The PCR products were ligated to TOPO TA pCR8 vector, and colony PCR showed 10 colonies out of 48 tested were correct in size (Fig.

13B). Sequencing results showed two clones (Bj2-PCR8-2,-7) sequences were correct and in reverse orientation for recombination reaction (Gateway vector system, LR reaction). The best matching clone (Bj2-PCR8-7) was used to recombine with SPP-pMDC100 vector, and was tested by colony PCR and restriction digestion (Fig. 13C, D). The plasmid of one of the resultant clones (Bj2-1) was used for *Agrobacterium* GV3101 transformation and was checked by colony PCR (Fig. 13E). It was named PS7 (SPP:Bj2/pMDC100, Fig. 13F) and was used for canola transformation.

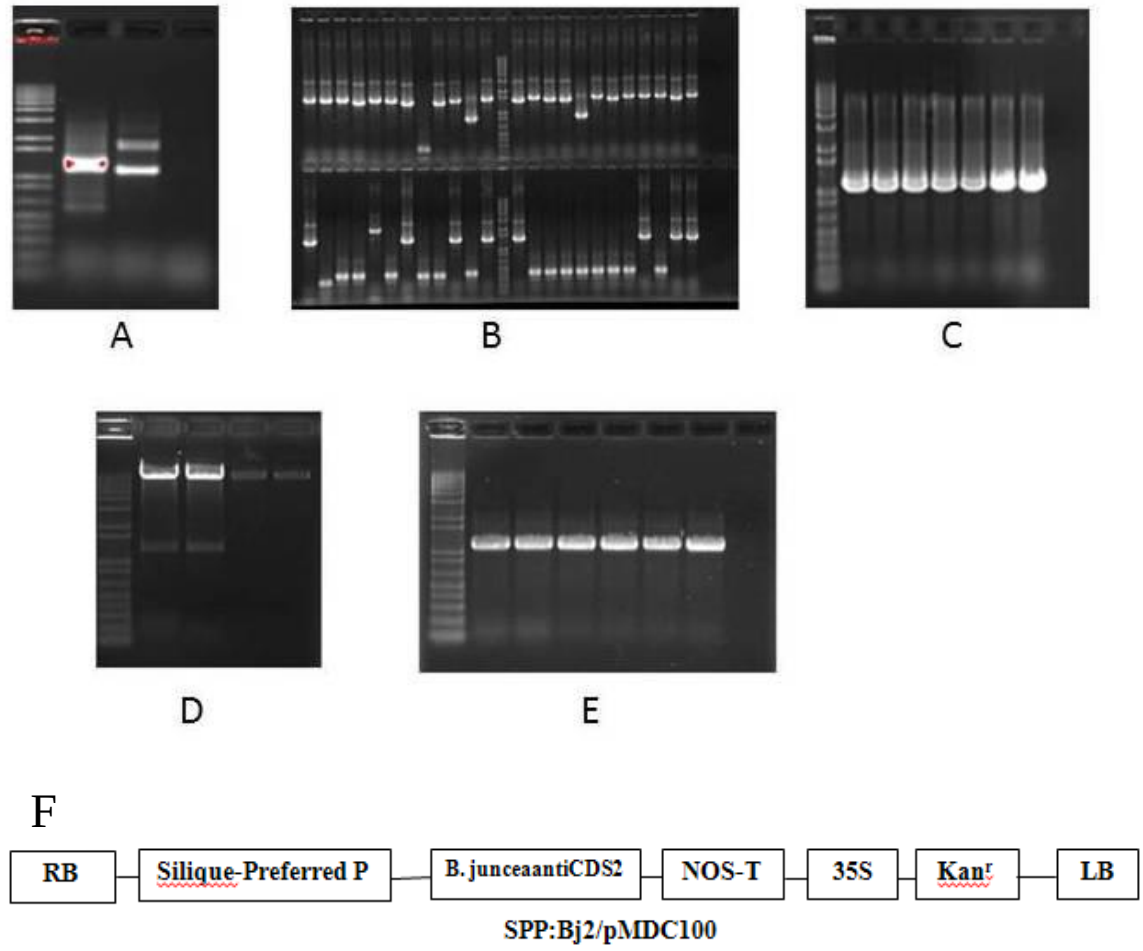
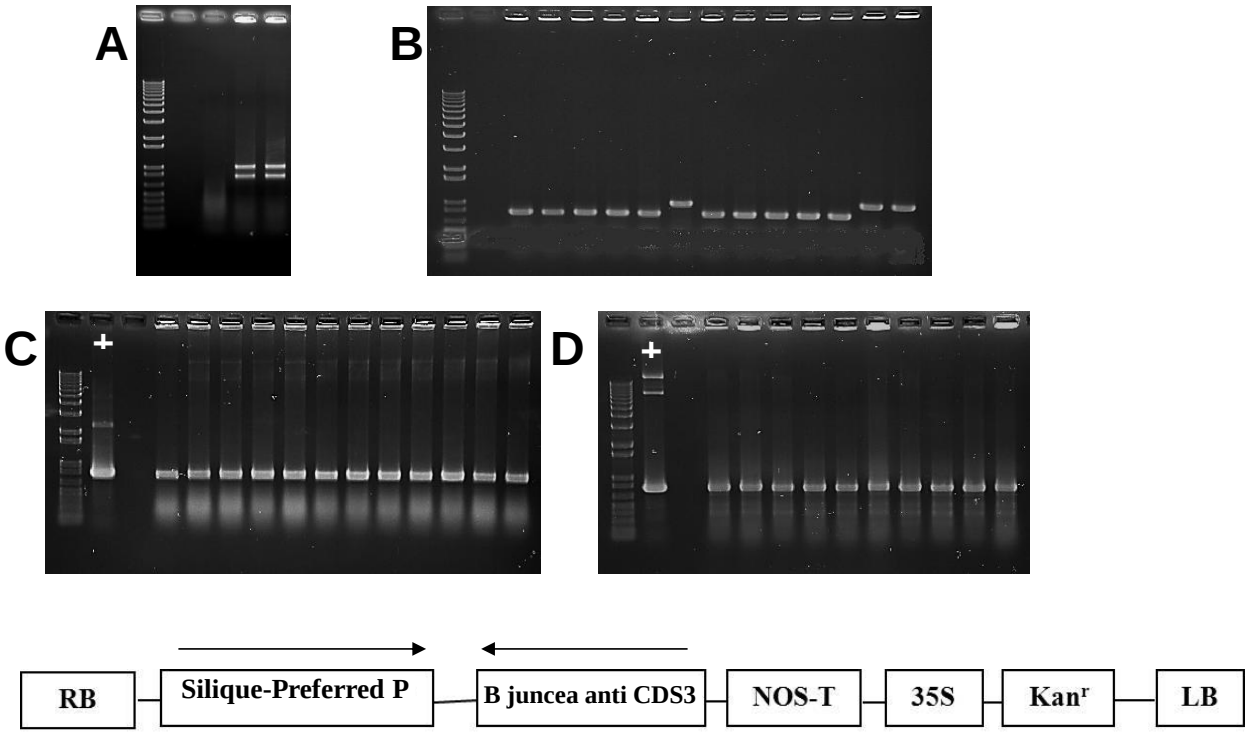


Figure 13: Preparing a gene construct (PS7) of *B. juncea* CDS *Bj2*, that is involved in pod shattering resistance, driven by a silique-preferred promoter

PS9 (SPP:Bj3/pMDC100): (Please note that we did not use PS8 and PS11 in this report) The expression pattern of Arabidopsis gene *At3* was similar to *AtIND1* gene, so this gene was selected for knockdown in canola to produce indehiscence canola. The *B. juncea* homolog (*Bj3*) of *At3* was amplified from *B. juncea* RNA by One-Step RT-PCR with gene specific primers, Bj3-F and Bj3-R. PCR produced two bands including the target band with the correct size (Figure 14). The PCR

products were ligated to TOPO TA pCR8 vector, and colony PCR identified 10 correct clones. Sequencing results confirmed two clones (Bj3-PCR8-2,-5) having correct DNA sequence and both were in reverse orientation for recombination reaction (Gateway vector system, LR reaction). The best matching one (Bj3-PCR8-5) was used to recombine with SSP-pMDC100-2 vector and was tested by colony PCR. The plasmid of one of the resultant clones (Bj3-1) was used for Agrobacterium GV3101 transformation, checked by colony PCR, named PS9 (SPP:Bj3/pMDC100) and was used for canola transformation.



SPP:Bj3/pMDC100

Figure 14: Preparing a gene construct (PS9) of Bj3 gene in the antisense orientation driven by silique-preferred promoter. A: Bj3 gene amplified from *B. juncea* RNA, B: Colony PCR of Bj3-PCR8-1:13, C: Colony PCR of Bj3-1:12 in *E.coli*, D: Colony PCR of Bj3-1:10 in *Agrobacterium* GV3101

PS10 (SPP:Bj4/pMDC100): The expression pattern of Arabidopsis gene *At4* was similar to *AtFULL*, so this gene was selected for overexpression in canola to produce indehiscence canola. The *B. juncea* homolog (*Bj4*) of *At4* was amplified from *B. juncea* RNA by One-Step RT-PCR with gene specific primers, Bj4-F and Bj4-R. PCR produced two bands including the target band with correct size (Figure 15). The PCR products were ligated to TOPO TA pCR8 vector, colony PCR identified 5 correct clones. Sequencing results confirmed three clones (Bj4-PCR8-1,-9, -12) having correct DNA sequence and all were in forward orientation for recombination reaction (Gateway vector system, LR reaction). The best matching one (Bj4-PCR8-1) was used to

recombine with SSP-pMDC100-2 vector and was tested by colony PCR. The plasmid of one of the resultant clone (Bj4-1) used for *Agrobacterium* GV3101 transformation, checked by colony PCR, named PS10 (SPP:Bj4/pMDC100) and used for canola transformation.

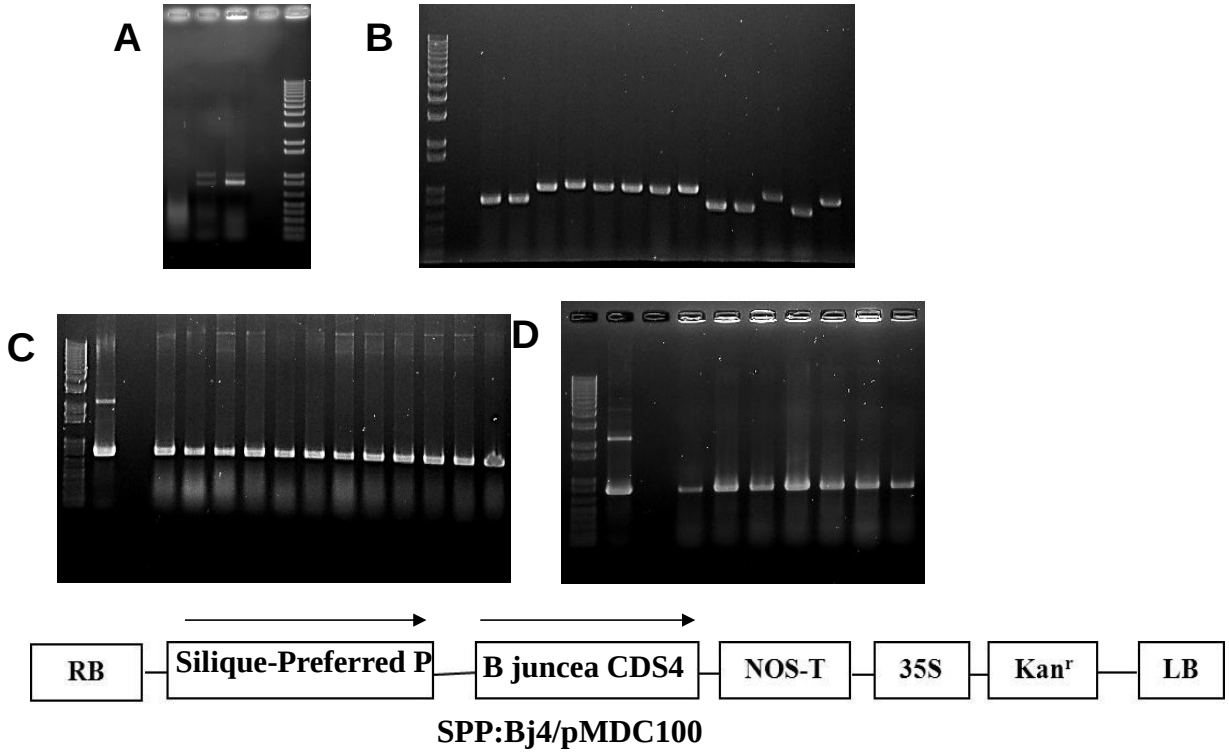


Figure 15: Preparing a gene construct (PS10) of Bj4 gene in the sense orientation driven by silique-preferred promoter. A: Bj4 gene amplified from *B. juncea* RNA, B: Colony PCR of Bj4-PCR8-1:13, C: Colony PCR of Bj4-1:12 in *E.coli*, D: Colony PCR of Bj4-4:10 in *Agrobacterium* GV3101

PS12 (SPP:Bj5/pMDC100): The expression pattern of Arabidopsis gene *At5* was similar to *AtFULL*, so this gene was selected for overexpression in canola to produce indehiscent canola. PBI EST database, Brassica Gateway BLAST and NCBI blast were used for homolog search. One homolog was found in *B. juncea* and this homolog (*Bj5*) was amplified from *B. juncea* RNA with gene specific primers Bj5-F and Bj5-R. The PCR product was ligated to TOPO TA pCR8 vector and sequenced to find a clone with correct identity and orientation. Clone 5, having the insert in forward orientation, was used to recombine with SSP-pMDC100 vector through Gateway vector system, LR reaction and was tested by colony PCR (Fig. 16). The plasmid of one of the resultant clones (Bj5-1) was used for *Agrobacterium* GV3101 transformation, checked by colony PCR, named PS12 (SPP:Bj5/pMDC100, Fig. 16) and is ready for canola transformation.

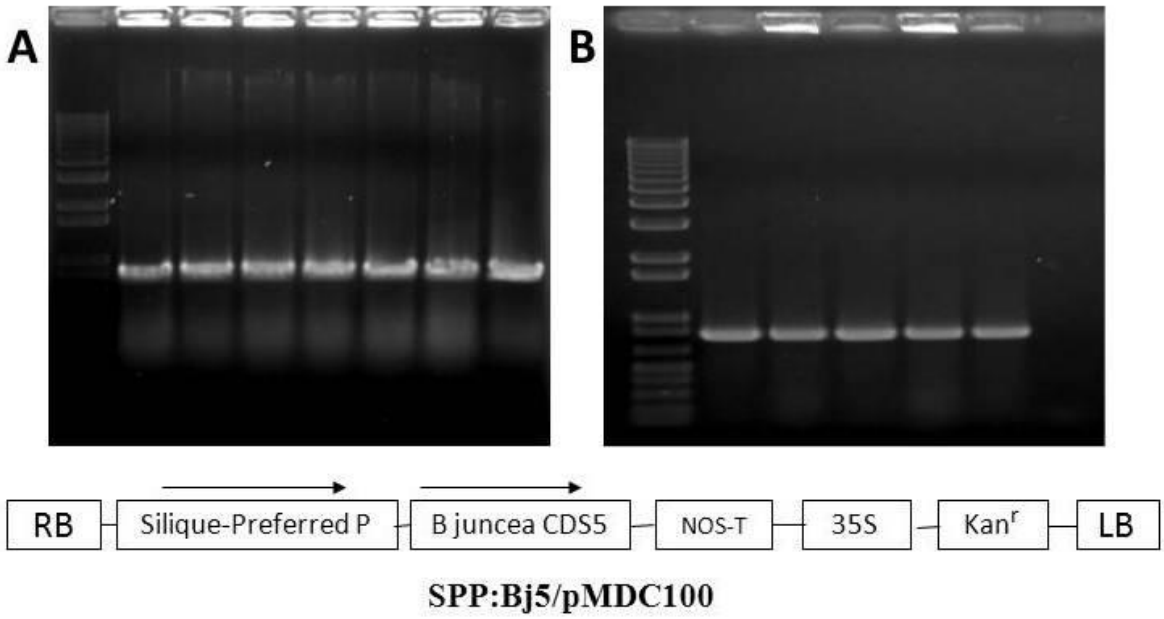


Figure 16: Preparing a gene construct (PS12) of Bj5 gene in the sense orientation driven by silique-preferred promoter. A: Colony PCR of pS12-1:7 in *E.coli*, B: Colony PCR of pS12-1:5 in Agrobacterium GV3101

5.5: Canola transformation:

Canola transformation was completed with most of the constructs (PS1, 2, 3, 5, 6, 7 and 9). Transformation with PS10 was not successful. Continuation of research is necessary for testing all the potential *B. juncea* genes in *B. napus* for their ability to reduce pod shattering. In total we raised 226 transformed shoots and transferred 170 shoots in soil (Fig. 17). Out of these, 144 plants produced flowers and pods.

Figure 17: Transformed canola shoots/plants

5.6: Fruit morphology:

Out of the 85 transformed shoots with PS1, PS2 and PS3, 56 plants produced successful fruits (Table 1). Among these, 33 plants produced normal, healthy and abundant pods (Fig. 18 and 19). This was a very encouraging result because we were able to select for plants with normal pods and some of these lines showed various degrees of pod shattering resistance as presented in the next section. From PS5, 6, 7 and 9 constructs, 42 plants produced normal pods (Table 1).

Table 1: Status of canola transformation with various pod shattering gene constructs. Numbers in the table represent the number of transformed plants at various stages.

Construct	Shoots produced in tissue culture	Plants in soil	Plants showed fruiting	Plants harvested; produced pods	Plants with normal pods
PS1	29	20	19	17	11
PS2	31	21	19	16	11
PS3	25	22	18	18	11
PS5	40	35	31	20	9
PS6	30	19	17	17	13
PS7	40	29	20	16	10
PS9	31	24	20	18	10
PS10	0	0	0	0	0
PS12	Started				
Total	**226**	**170**	**144**	**122**	**75**

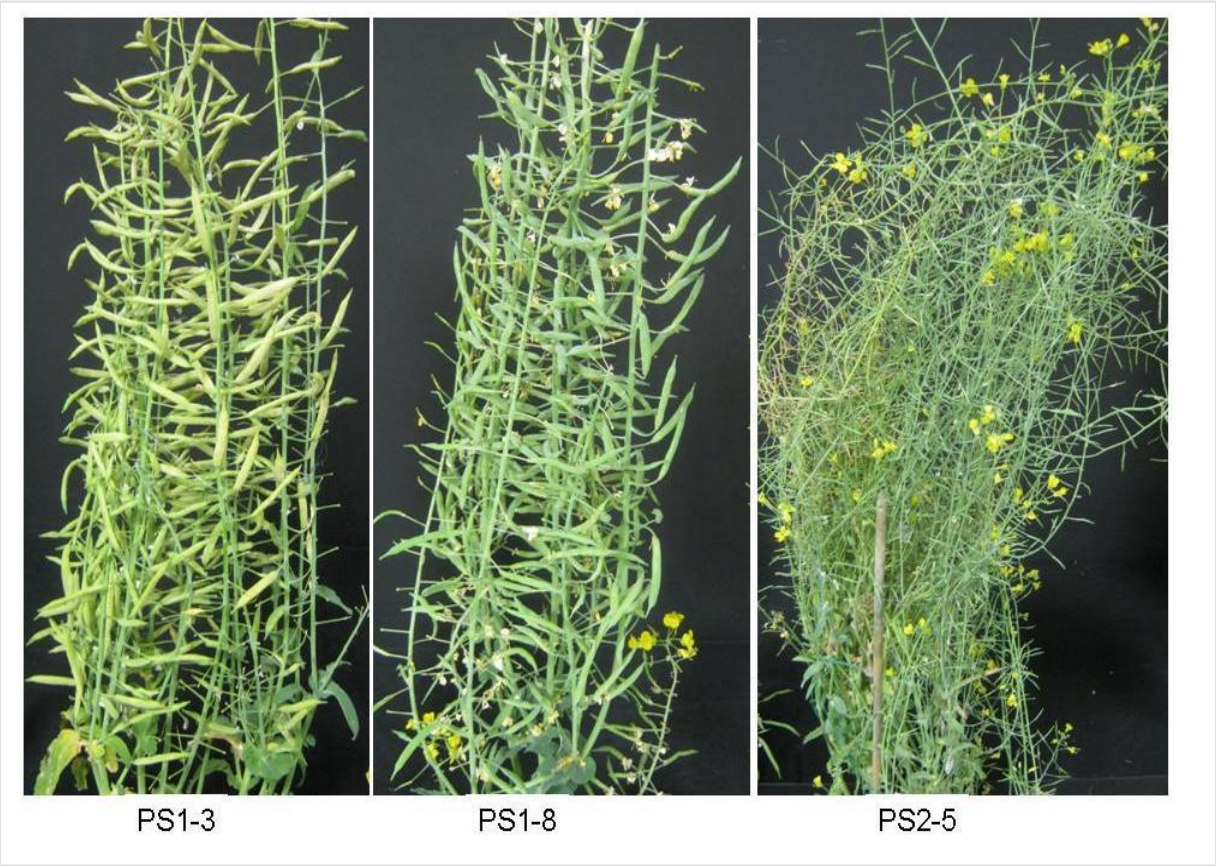


Figure 18: Examples of transformed plants with PS1, PS2 and PS3 constructs. Out of 56 plants producing pods from these constructs, 33 produced normal abundant pods like those two plants in the left panels

Figure 19: Example of few canola lines transformed with PS constructs. DH is non-transformed control

It may be mentioned here that the pod morphology might have also been influenced by the tissue culture process and hormones in the growth medium. Therefore, the potential lines with pod shattering resistance were grown from seeds for the next generation; and we analyzed these plants and their pods for shattering resistance to confirm that the trait inherits in the subsequent generations (see next sections).

5.7: Analysis for pod shattering resistance (first generation):

Pod shattering resistance assessment of first generation T_0 transformed canola lines were completed for PS1 through PS7. We have optimized a process of bringing moisture content in all samples to an almost equal level (~7%). This was done by leaving the harvested pods at room temperature and ~30 relative humidity for at least one month; followed by keeping the pods in a controlled environment chamber in dark at 50% relative humidity and 20^0 C for 7 days. We have also optimized a method to assess pod shattering in our laboratory settings by shaking the pods with metal rods or metal beads (ball bearings; 4 -8 mm diameter, 0.5-4.0 gm) in polypropylene tubes on a reciprocating shaker. Many variables were tested for this as shown in Table 2. The best and most consistent results were obtained by using 10 pods in a 250 ml polypropylene tube with one metal rod piece (17 mm long, 7 mm diameter, 7 gm weight) placed vertically flat (right angle to the path of shaking) on the surface of the reciprocating shaker and shaken for 15 seconds at 400 rpm. The non-transgenic double haploid canola line DH12075 (DH) was used in these optimization experiments.

Table 2: Factors and variables involved in the optimization of pod shattering assessment using the non-transgenic DH12075 (DH) canola fruits.

Factors	Variables
Pod numbers	5 or 10 pods
Number of repeats	3 to 5
Metal rods number	1 to 4 pieces
Metal beads number	1 to 6
Tube size	50 ml or 250 ml tubes
Time	15, 30, 45, 60, 90, 120, 180, 240, 360 & 420 seconds
Speed	200 or 400 rpm
Tube position on the shaker	Vertical or horizontal (both flat)*

*Vertical/horizontal positions are in relation to the path of shaking.

At the above mentioned conditions, 60-70% pods of the non-transgenic DH line shattered, compared to 30-40% of *Brassica juncea* and 10% of *Sinapis alba*, as expected. Therefore, these conditions were used to analyze transgenic lines for pod shattering resistance, along with non-transgenic controls (Table 3). Transgenic PS1, PS2 and PS3 lines producing normal looking pods that showed ≤40% shattering were selected to raise the second generation plants in greenhouses.

Table 3: Pod shattering resistance assessment for first generation transgenic canola lines (PS1, PS2, PS3 and PS6) transformed with Arabidopsis gene constructs compared to non-transgenic DH, *Brassica juncea* and *Sinapis alba* as controls.

Name	No. of lines analyzed	No. of observations /line	Pod shattering (PS) range (%)*	No. of lines with ≤40% pod shattering	No. of lines with normal looking fruits among the ≤ 40% PS	Moisture content range (%) (Mean)
PS1	17	3	10-80	6	4	6.0-8.3 (7.2)
PS2	16	3	0-80	10	7	6.8-7.6 (7.2)
PS3	18	3	0-85	4	2	6.6-7.7 (7.2)
PS6	16	5	15-70	5	2	-
DH	3	3	60-70	0	-	7.6-7.7 (7.6)
B. juncea	3	3	30-40	3	3	7.2-7.5 (7.3)
S. alba	3	3	10	3	3	-

* % pods completely shattered at optimized conditions described in the text.

Out of seven potential *B. juncea* genes, we have completed transformation of *B. napus* canola with three gene constructs (PS5, PS7 and PS9) and finished pod shattering assessment of two of them (PS5 and PS7). Among PS9 lines, one was ready for shattering assessment during the preparation of this report. Pod shattering assessment results of canola lines transformed with *B. juncea* gene constructs are shown in Table 4. Several lines showed reduced pod shattering with normal pods in the first generation. We would like to test all the identified *B. juncea* genes in canola and confirm the pod shattering resistance of potential lines in subsequent generations.

Table 4: Pod shattering assessment for first generation transgenic canola lines (PS5, PS7, PS9 and PS12) transformed with *B. juncea* gene constructs compared to non-transgenic DH and *B. juncea*.

Name	No. of lines analyzed	No. of observations /line	Pod shattering (PS) range (%)*	No. of lines with ≤40% pod shattering	No. of lines with normal looking pods among the ≤ 40% PS
PS5	9	3-5	10-85	3	2
PS7	9	5	5-80	4	2
PS9	1	5		1	1
DH	3	5	50-60	0	-
B. juncea	2	5	25-30	2	2

5.8: Analysis for pod shattering resistance (second generation):

Transgenic PS1 - PS3 lines which showed ≤40% pod shattering in the first generation were grown in the greenhouse and their pods were analyzed for shattering (Table 5). We have identified 26 lines from the three constructs which showed ≤20% pod shattering (29 lines showed ≤30%) compared to ~55% in the control DH line. Most of these pod shattering resistant lines had normal pods and normal seed set (Table 5). They were grown again for raising homozygous lines and for increasing their seeds for field trial.

Table 5: Pod shattering resistance assessment for second generation transgenic canola lines (PS1, PS2 and PS3) transformed with 3 gene constructs compared to non-transgenic DH and *Brassica juncea* controls.

Name	No. of family tested	No. of lines/ family	Pod shattering range (%)	No. of lines with ≤20% pod shattering	Fruit length (cm)*	Fruit no/plant*	Seed no/fruit*	Yield/plant *
PS1	4	48	0-56	4	6.7 - 7.6	263 - 418	19 - 29	17.7 – 29.1
PS2	7	104	0-56	15	6.4 – 7.9	167 - 435	22 - 31	12.8 – 30.4
PS3	2	24	0-56	7	6.0 – 7.5	176 - 425	22 - 26	12.2 – 31.5
DH	-	5	46-68	0	6.5 – 7.3	209 - 285	24 - 27	15.8 – 22.9
B. juncea	-	5	3-13	5	-	-	-	-

*, of the lines which had ≤ 20% pod shattering and were selected for field trial.

The result of selection for pod shattering resistance were very encouraging. In the first generation, the transgenic lines showed 0 to 85% shattering (Figure 20A). Those with 40% or less pod shattering were moved to the second generation; and in the second generation they showed 0 to 56% shattering (Figure 20B). This shift in pod shattering suggests that this trait in our transgenic lines is due to the effect of the introduced genes; and not random. It may be mentioned here that few second generation lines were still segregating for the transgene; and thus produced some lines which showed more than 40% shattering.

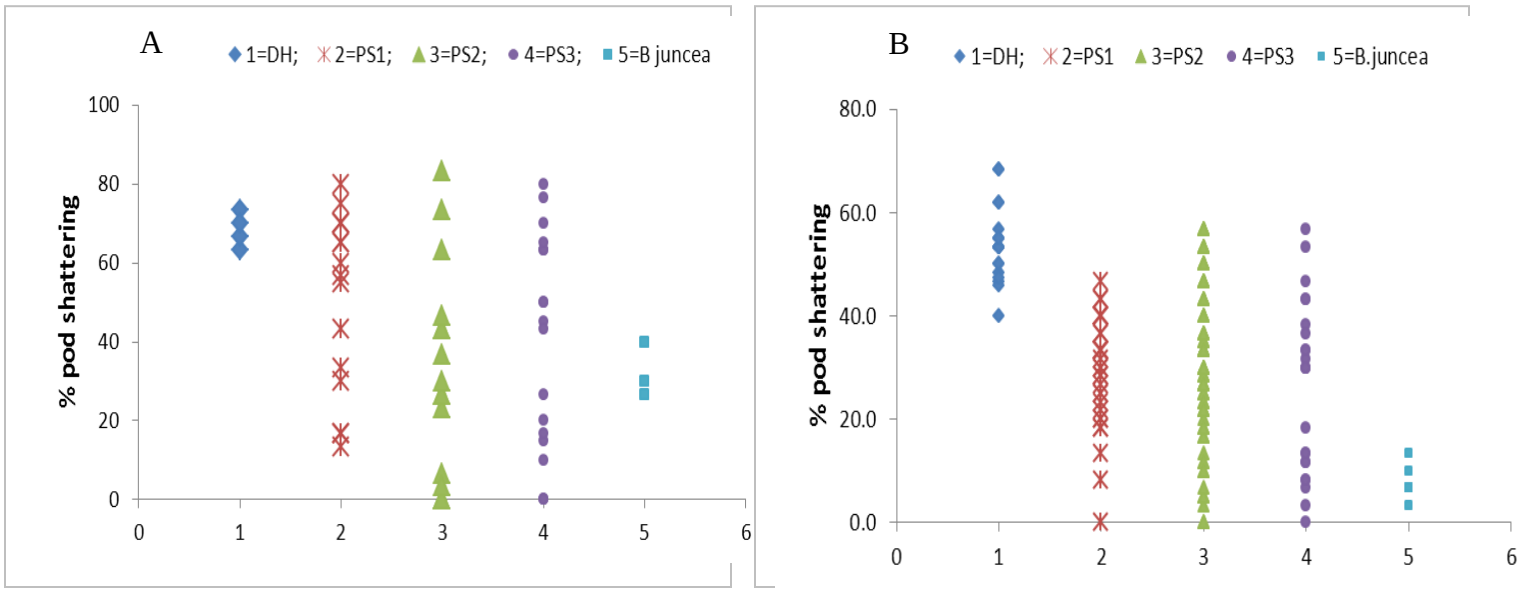
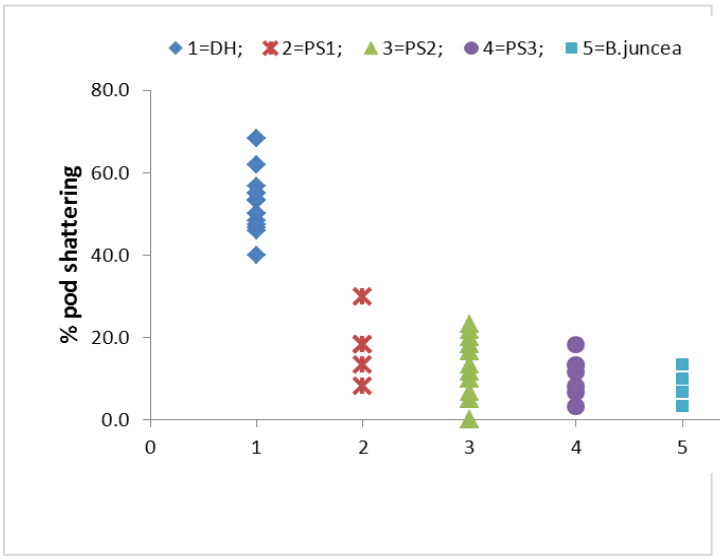
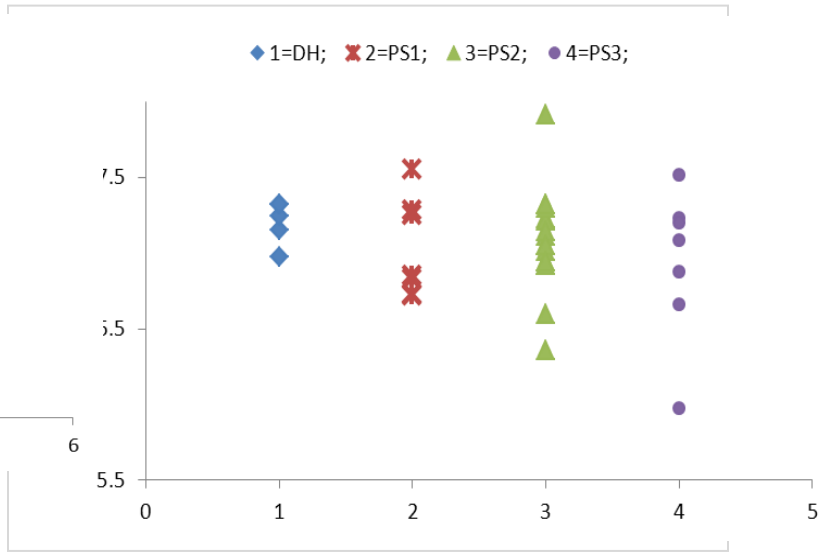
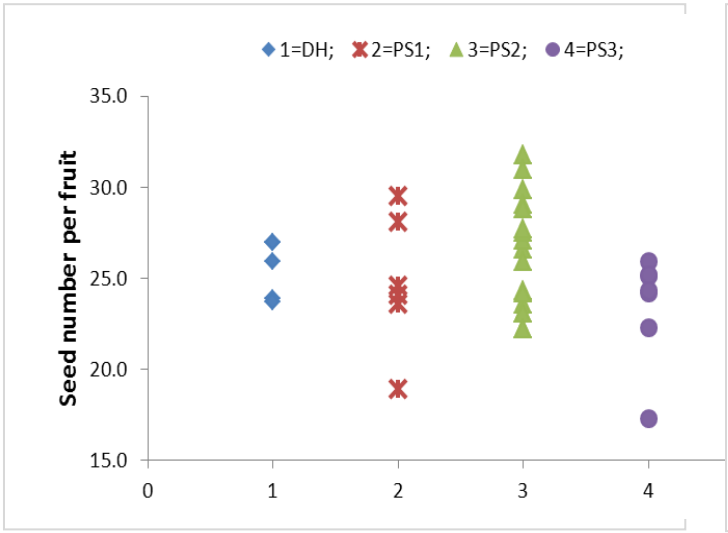
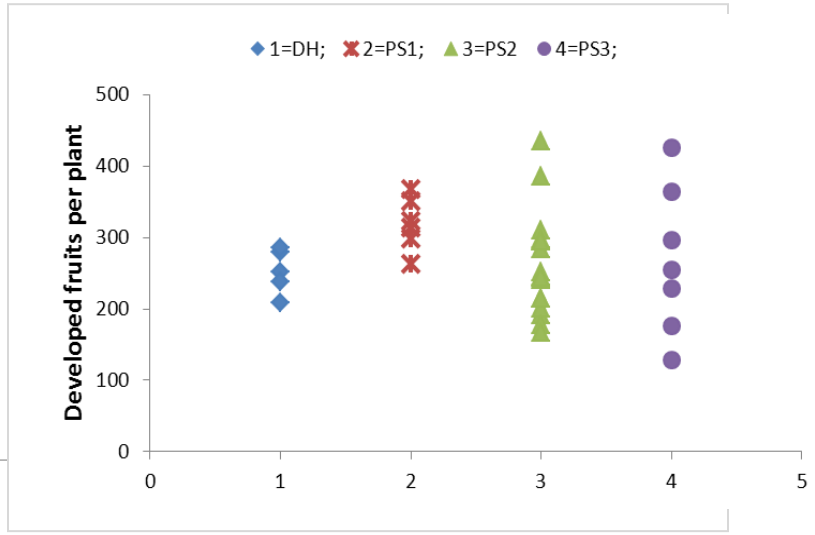


Figure 20: Pod shattering in first (A) and second (B) generations of transgenic canola lines compared to non-transformed DH canola.









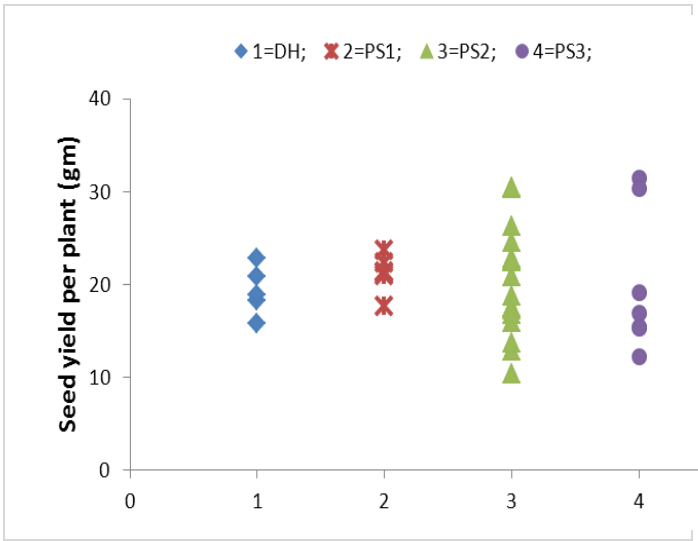
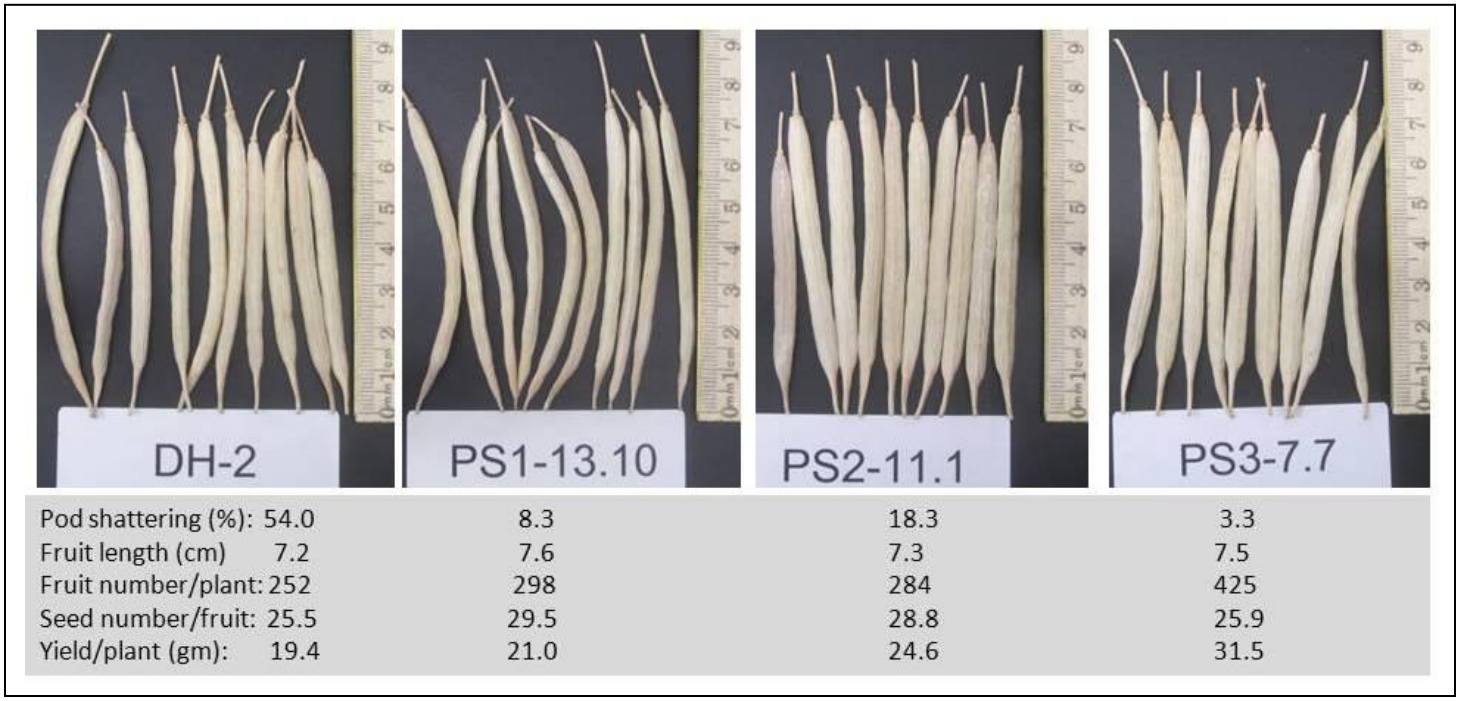


Figure 21: Pod shattering and other agronomic traits of transgenic canola lines selected for field trial, compared to non-transformed DH canola

In respect to agronomic traits recorded from the greenhouse grown plants, most of the transgenic lines selected for the field trial had similar agronomic performance like the non-transformed DH control (Fig. 21). Three examples of lines selected for field trial are shown in figure 22.



	DH-2	PS1-13.10	PS2-11.1	PS3-7.7
Pod shattering (%):	54.0	8.3	18.3	3.3
Fruit length (cm)	7.2	7.6	7.3	7.5
Fruit number/plant:	252	298	284	425
Seed number/fruit:	25.5	29.5	28.8	25.9
Yield/plant (gm):	19.4	21.0	24.6	31.5

Figure 22: Examples of three lines selected for field trial; and their agronomic traits compared to non-transformed DH canola

5.9: Field trial:

We conducted a CFIA (Canadian Food Inspection Agency) authorized confined research field trial with promising lines (which showed reduced pod shattering in lab tests), along with non-transformed DH12075 control (DH), in AITF's field at Vegreville, AB. As anticipated, we faced several challenges in the field trial, e.g., (1) we tried to have equal number of plants in each plot to eliminate the effect of plant density on pod shattering (see materials and methods, Fig. 34). However, it was difficult to achieve this due to various environmental factors including diseases; (2) ideally, we were hoping that all plots would mature at the same time. However, there was slight difference in the maturity date between plots and even between plants within a plot, which was partly due to non-synchronized seed germination in the field. Therefore, we waited for fall freezing so that all plots freeze to equal level for pod shattering. The plots did freeze during the first week of October 2013, and we had to harvest the field in cold weather. We are not sure

about the effect of freezing on fully and partially matured pods on shattering; (3) on October 16th we harvested two replications; and that night and the next morning there was a strong wind that affected pod shattering. We harvested other two replications on October 17th; (4) there was not enough natural pod shattering. Therefore, we had to induce pod shattering by shaking the plants. We tried to apply equal shaking to each plot, but human error could not be eliminated; (5) we placed four trays in each plot to collect seeds fallen from shattered pods (see materials and methods, Fig. 35 and 36). The area of these four trays represents only 1/8.83 of the entire plot. Therefore, data obtained from trays were extrapolated by multiplying with 8.83 to obtain data for the whole plot. By doing so, any small error might have been expanded; (6) although we placed the four trays in the same spots in each plot, there was no guarantee that those spots represent that plot in respect of plant density; etc. These difficulties might have introduced greater variability and some errors in field data; and should be considered when interpreting the field results.

5.9.1: Pod shattering in the field trial: Canola lines tested in the field are shown in Table 6. Most of these lines were homozygous and showed less than 30% pod shattering in the lab test (except line 6 which showed 40% shattering), compared to 55% in DH control. In the field trial, however, only some (not all) of the lines showed reduced pod shattering compared to the DH control (Fig. 23). Percent seed loss before combining (BC, natural loss plus those from pod shatter induction) and during straight cut combining (DC) were added to calculate total loss (BC+DC, Fig. 23). The best performing line (line #25) had 31% total seed loss compared to 44% in DH control. This represents 30% less pod shattering than DH control in the field. In lab tests, this particular line #25, as an example, showed 13% shattering compared to 56% in DH control (which is 75% less shattering than DH). This was noticed in other lines too; differences between transgenic lines and DH control was much less in the field trial than in lab tests. Moreover, variation between replications was higher in the field trial (Fig. 24). For example, line 7 in four replications showed 46, 118, 47 and 82 percent of control DH's seed loss. We believe that some of the challenges faced in the field trial might have contributed errors in data, and therefore, further field trials are recommended, preferably in locations where pod shattering trait expresses naturally.

We noticed that, along with seed drop in the trays, some non-shattered whole pods dropped from the plants (Fig. 25). We collected both seeds and these pods from the trays and analyzed

separately. In some lines, such as line 25, dropped pods contributed substantially in total seed loss (Fig. 26). If only seed drop is considered, this line showed 68% less loss than DH control (compared to only 30% when pod drop is included). It is possible that shattering resistant pods do not open easily, and therefore break off from the plants when subjected to strong wind and shaking.

Table 6: Canola lines tested in the field trial. Line 31, DH12075 (DH), is non-transformed control.

Line number	Line name	Segregation ratio	Estimated Copy #		DF*	sd
1	PS1-13.10	20:0	1		48.25	0.50
2	PS1-17.7.8	20:0	1		50.00	0.00
3	PS1-19.1	20:0	1		49.25	0.50
4	PS1-20.7	20:0	1		49.00	0.00
5	PS1-20.8	14:6	1		49.75	0.50
6	PS1-20.12	20:0	1		49.00	0.00
7	PS2-8.10	20:0	2		48.75	0.50
8	PS2-8.11	20:0	2		49.25	0.50
9	PS2-10.8	20:0	2 or 3		48.50	0.58
10	PS2-10.10	20:0	2 or 3		48.25	0.50
11	PS2-10.16	20:0	2 or 3		48.50	0.58
12	PS2-11.1	20:0	2 or 3		48.50	0.58
13	PS2-11.3.3	20:0	2 or 3		48.25	0.96
14	PS2-12.4	20:0	1 or 2		48.50	0.58
15	PS2-12.9	20:0	1 or 2		49.25	0.50
16	PS2-12.14	20:0	2		49.00	0.82
17	PS2-13.4	20:0	>2		48.00	0.00
18	PS2-13.9	20:0	>2		48.50	1.00
19	PS2-13.10	20:0	>2		47.75	0.50
20	PS2-14.6	20:0	1 or 2		48.00	0.00
21	PS2-14.12	20:0	1 or 2		49.50	0.58
22	PS2-18.1	20:0	1		49.00	0.00
23	PS2-18.10	20:0	1		48.50	0.58
24	PS3-1H.8.9	20:0	1		50.25	0.50
25	PS3-1H.12	20:0	1		49.25	0.50
26	PS3-7H.2	19:1	2		48.50	0.58
27	PS3-7H.3.2	20:0	2 or 3		49.25	0.50
28	PS3-7H.7	20:0	3		48.50	0.58
29	PS3-7H.9	20:0	2 or 3		48.75	0.50
30	PS3-7H.12	17:3	2 or 3		48.75	0.96
31	DH 12075	0:20	0		48.25	0.50

*DF, days required for 10% plants to flower; sd, standard deviation. Segregation ratio was calculated from the 'embryo assay' as mentioned in the materials and methods. Estimated transgene copy number in each canola line is shown.

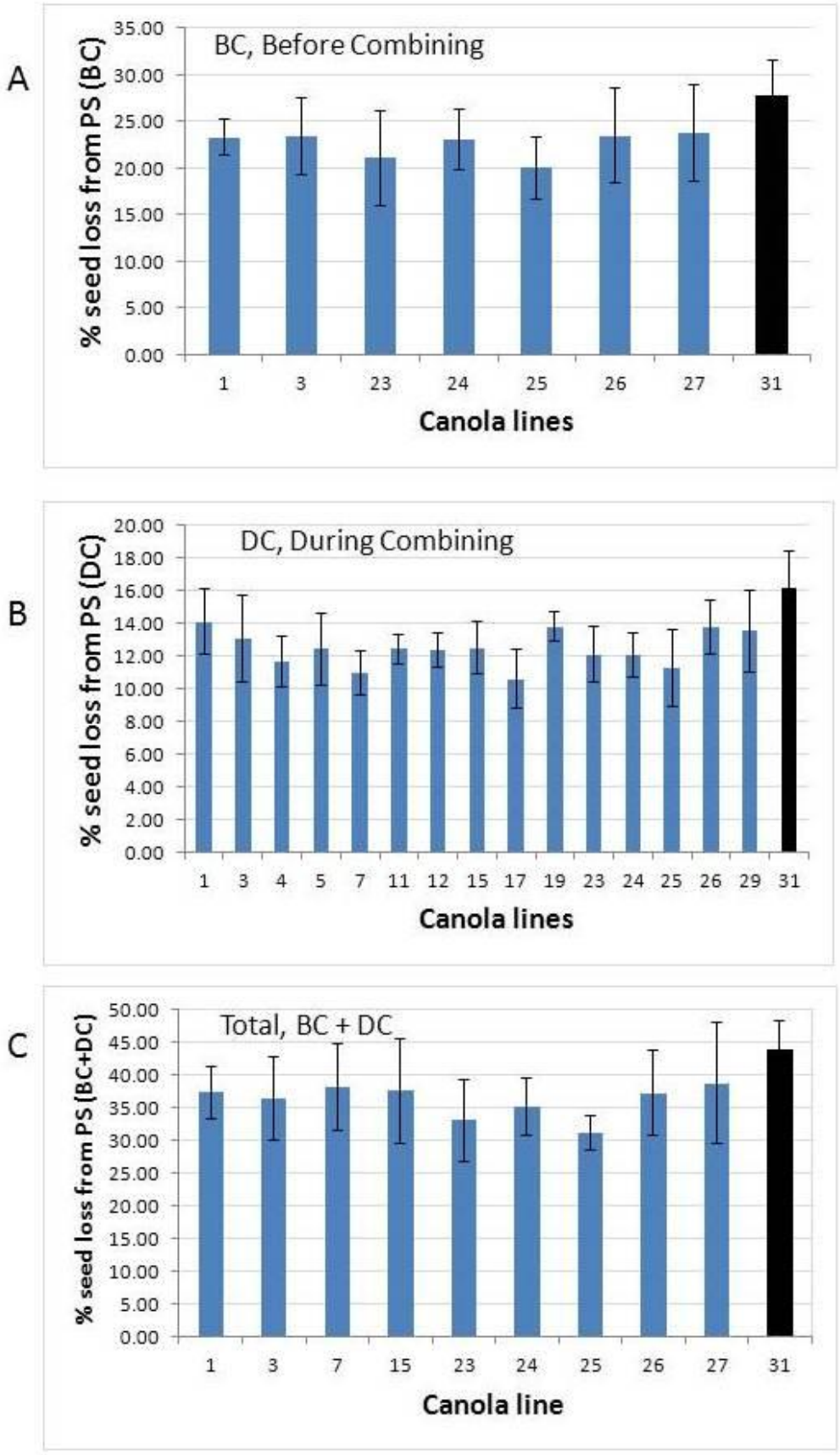


Figure 23: Seed loss (%) in field trial. Loss in transgenic lines (blue) is compared to non-transformed DH control (line 31, black). Error bars are standard deviations

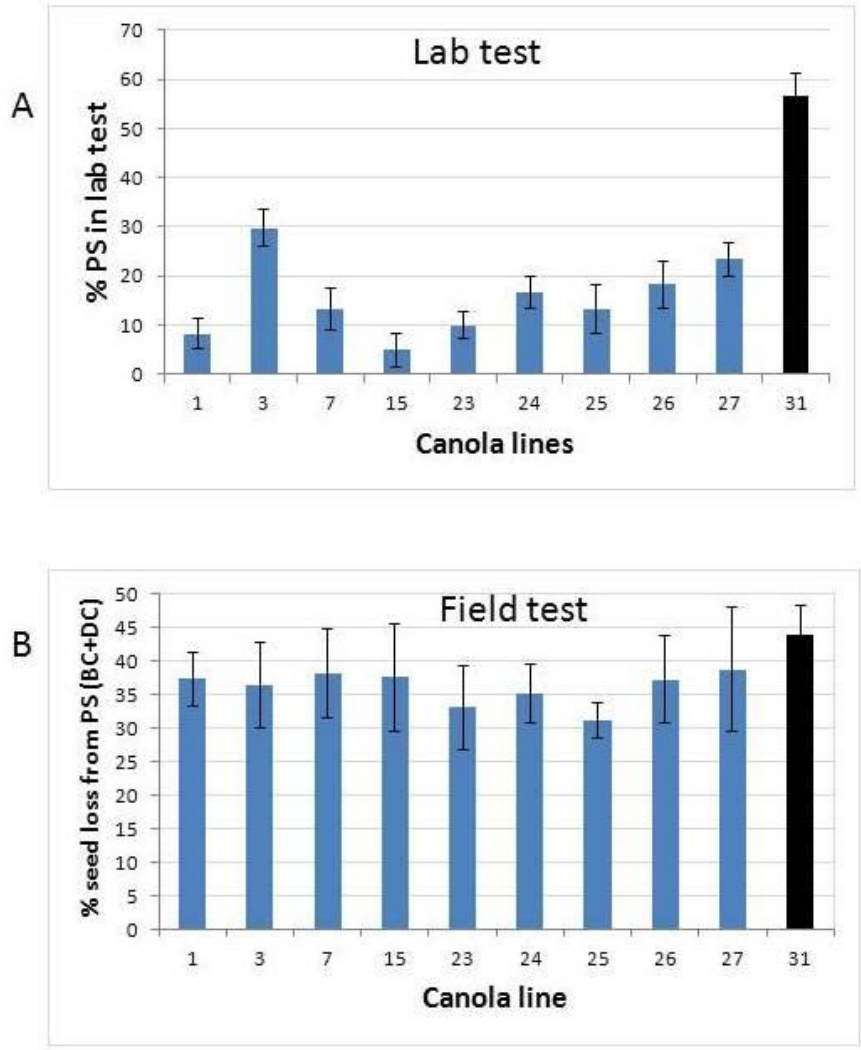


Figure 24: Seed loss (%) in transgenic lines (blue) compared to non-transformed DH control (line 31, black) in lab test vs. field test.

Figure 25: Example of what was collected in the trays placed in the field plots

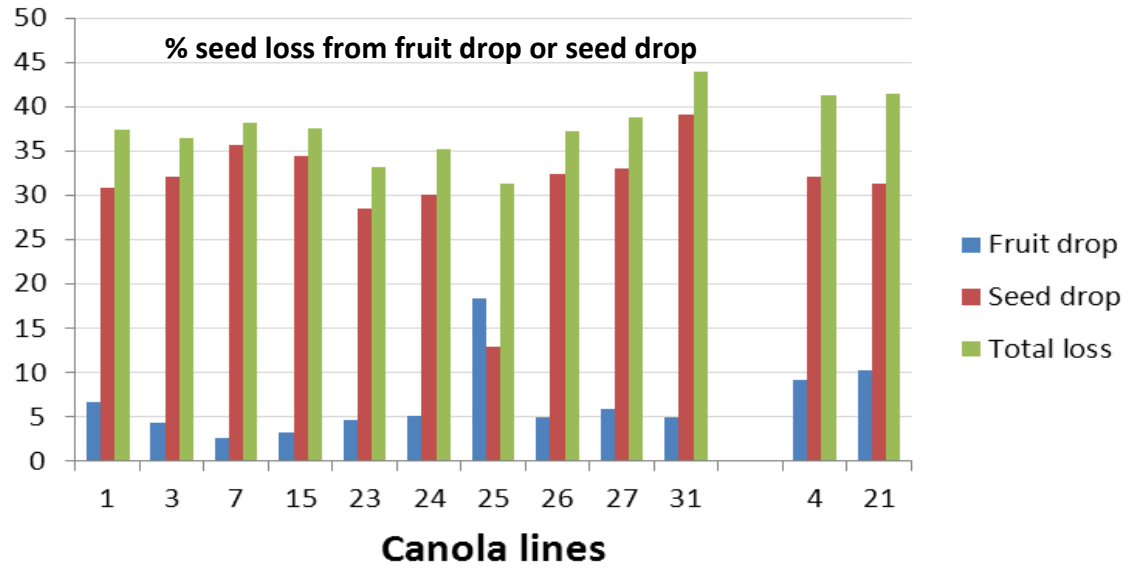


Figure 26: Total seed loss (%) from fruit (pod) drop or seed drop in field trial. Line 31 is non-transformed DH control

5.9.2: Agronomic traits of field grown plants: We did not notice any obvious differences in plant and pod morphology among the transgenic lines, nor between transgenic lines and non-transformed DH control. In respect to flowering date, all lines flowered 48-50 days after seeding (Table 6). Detailed agronomic data for 10 transgenic lines, along with the DH control, is shown in figures 27 and 28. As expected, there was a large variation within lines for most of the agronomic traits, but there was no noticeable difference between lines. Also, the pod shattering resistant and susceptible (in field test) lines showed a similar range in these agronomic traits (Fig. 28). Pod morphology of some lines is shown in figure 29 as examples. These results shows that selection of plants with normal pods among the shattering resistant lines was effective in developing lines with reduced pod shattering and normal agronomic traits. However, we recognize that this selection might have compromised improvement in pod shattering resistance to some extent.

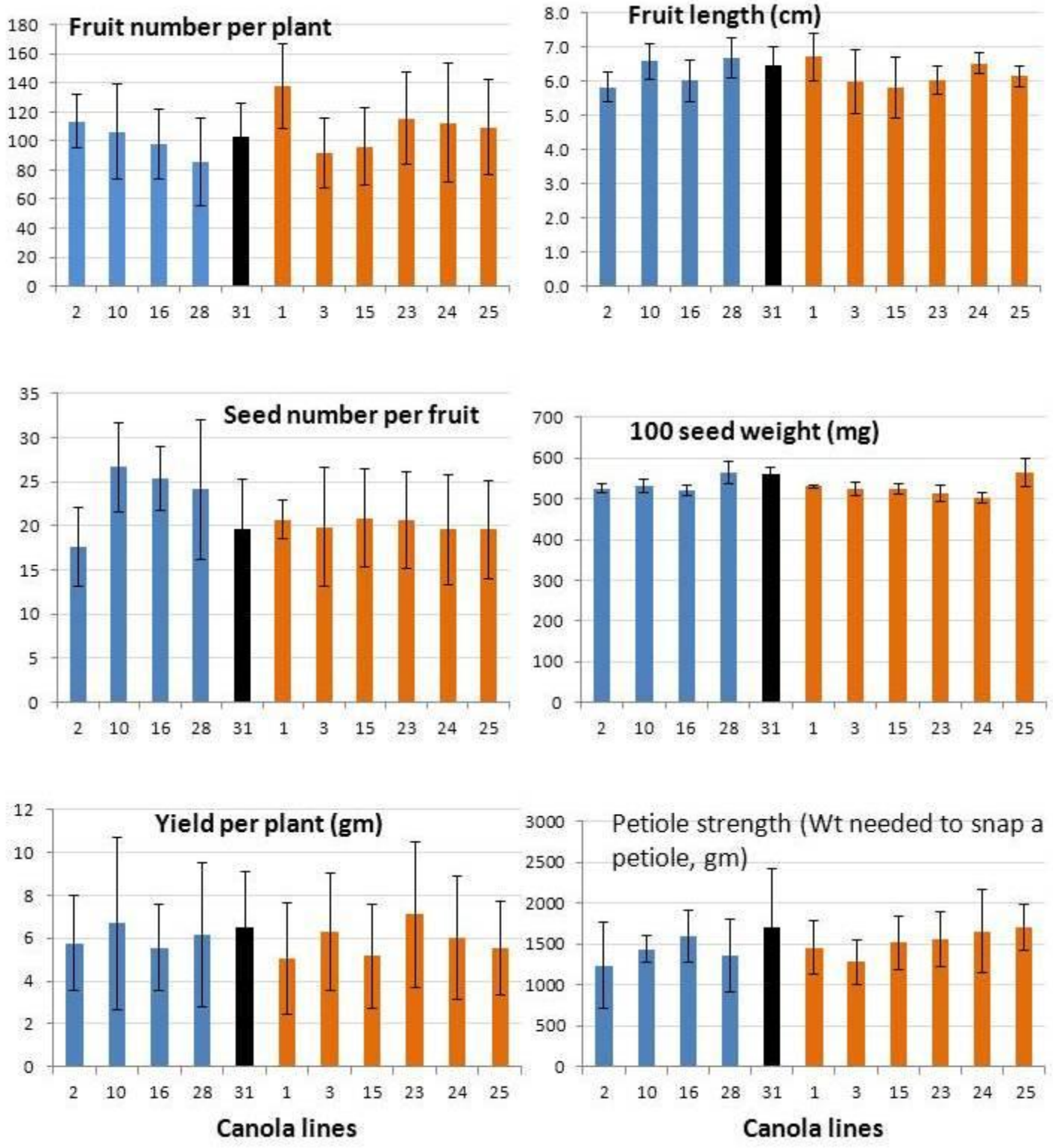


Figure 27: Data for some agronomic traits of transgenic canola lines compared to non-transformed DH control (line 31, black bar). In addition to DH control; lines 2, 10, 16, and 28 (blue bars) did not show pod shattering resistance in field trial. Other lines (1, 3, 15, 23, 24 and 25; orange bars) showed various degrees of shattering resistance in the field

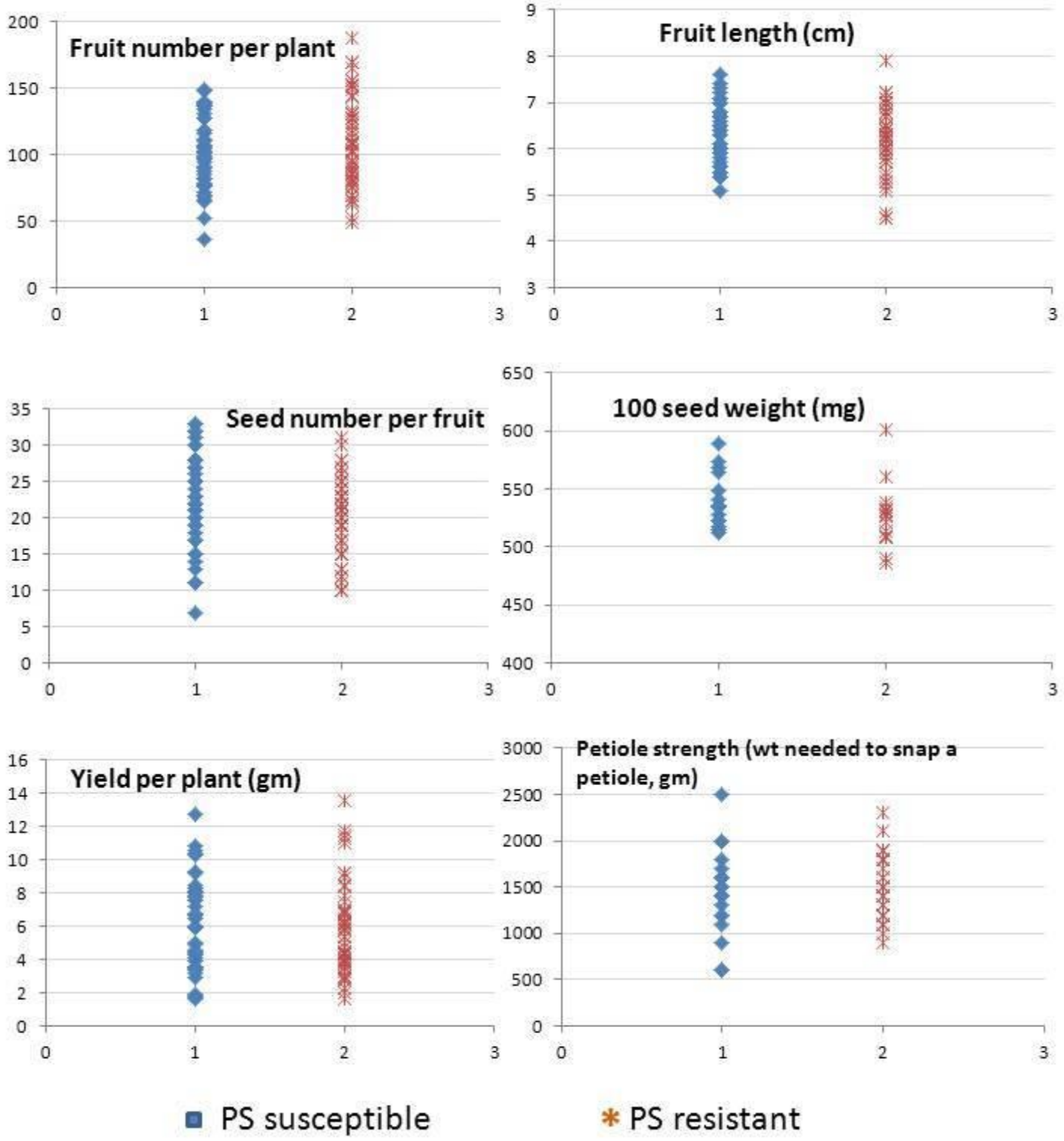


Figure 28: Comparison between lines which showed pod shatter (PS) resistance (orange) in field and those which did not show such resistance (blue) in respect to several agronomic traits

Figure 29: Pods of canola lines which showed various degrees of shattering resistance in the field trial (top two rows) compared to those which did not show such resistance (bottom two rows). Line 31 is non-transformed DH control

5.9.3: Seed oil analysis: Oil content in mature seeds of selected (those which showed pod shattering resistance and those which did not show such resistance in field) transgenic lines and non-transgenic DH control (line 31) is shown in figure 30. Oil content in these lines was very similar, ranging from 39 to 42%. In line 25 oil content was 39%, and in line 3 it was 42%. Non-transgenic DH control had 41% oil content.

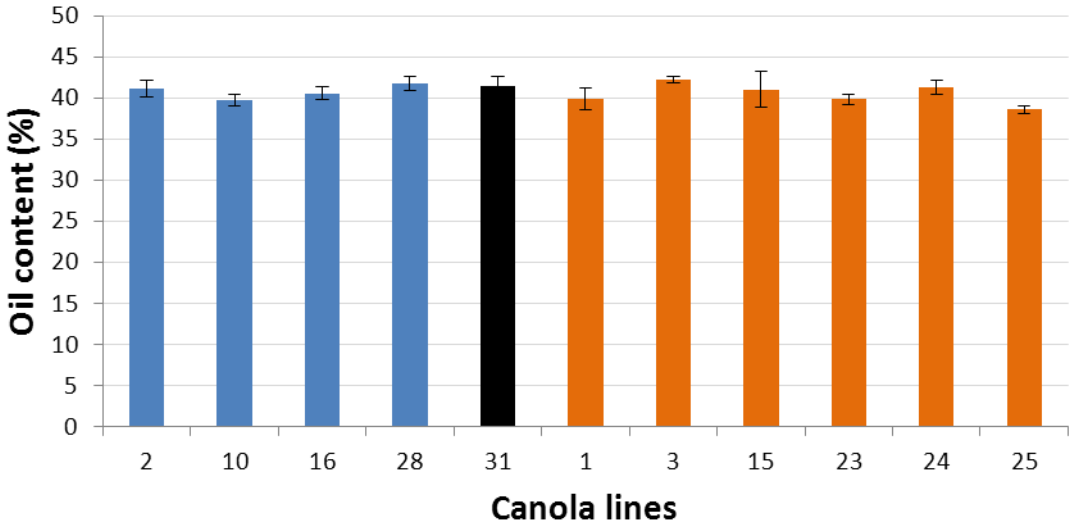


Figure 30: Seed oil content of transgenic and non-transformed DH control. Line 31 (black bar) is DH control. Orange bar lines showed reduced pod shattering in field, while blue bar lines did not show such reduction

Fatty acid content of seed oil of selected transgenic lines, along with non-transgenic DH control is shown in table 7. The only notable change was in line 25 where there was ~4% decrease in oleic acid (18:1Δ^{9}) with an equal increase in linoleic acid (18:2 $\Delta^{9,12}$).

Table 7: Fatty acid content (mean±standard deviation) of seed oil of selected canola lines. Line 31 is non-transformed DH control

Line	16:0	16:1	18:0	18:1 Δ^{9}	18:2 $\Delta^{9,12}$	18:3 $\Delta^{9,12,15}$	20:0	20:1 Δ^{11}	22:0	24:0
1	3.6±0.1	0.17±0.01	2.2±0.06	63.6±0.8	14.7±0.6	12.1±0.3	0.81±0.03	1.69±0.06	0.47±0.02	0.31±0.03
2	3.4±0.1	0.18±0.03	2.3±0.06	63.3±0.5	15.0±0.5	12.9±0.8	0.53±0.04	1.68±0.03	0.48±0.06	0.33±0.09
3	3.6±0.3	0.18±0.02	2.2±0.34	62.3±3.1	16.3±3.5	11.9±0.3	0.76±0.07	1.59±0.02	0.44±0.02	0.29±0.02
10	3.7±0.1	0.18±0.01	2.3±0.14	63.2±0.9	14.6±0.6	12.3±0.6	0.82±0.05	1.58±0.03	0.49±0.04	0.35±0.06
15	3.6±0.1	0.20±0.01	2.2±0.13	63.9±0.3	14.5±0.6	12.0±0.4	0.79±0.02	1.63±0.06	0.48±0.01	0.30±0.03
16	3.8±0.1	0.21±0.02	2.2±0.16	64.6±2.5	14.7±0.9	11.9±0.5	0.78±0.03	1.57±0.04	0.46±0.01	0.29±0.01
23	3.6±0.1	0.18±0.01	2.3±0.09	62.7±1.0	14.4±0.4	13.3±0.7	0.79±0.04	1.53±0.03	0.46±0.01	0.30±0.03
24	3.5±0.1	0.17±0.01	2.3±0.15	64.2±0.9	14.5±0.5	12.1±0.5	0.77±0.04	1.53±0.02	0.42±0.01	0.25±0.03
25	3.9±0.2	0.19±0.01	1.8±0.27	59.7±0.8	19.2±0.5	11.7±0.7	0.63±0.02	1.62±0.04	0.41±0.01	0.30±0.03
28	3.6±0.1	0.19±0.01	2.3±0.11	63.4±1.2	15.0±1.0	11.9±0.2	0.78±0.04	1.55±0.03	0.47±0.04	0.32±0.04
31	3.8±0.1	0.19±0.01	2.5±0.37	64.1±0.4	14.4±0.2	11.6±0.5	0.83±0.04	1.58±0.03	0.48±0.02	0.31±0.04

5.9.4: Molecular analysis: Figure 31 shows genomic PCR results of canola lines grown in the field. All the lines showed amplification of the correct size PCR products, compared to no amplification in the non-transformed DH control (line 31), confirming the transgenic nature of the lines tested in the field.

Southern analysis of selected canola lines showed integration of one to three copies of the transgene (Fig. 32). RT-PCR results showed expected suppression of the mRNA in two antisense lines (PS1 and PS2), while production of Arabidopsis FUL mRNA in the overexpression canola lines (PS3) (Fig. 33). Successful expression of the Arabidopsis transgenes in canola in the expected pattern was reflected in reduced pod shattering in lab tests, but not in some lines in the field (e.g., lines 2, 10, 16 and 28). This observation further confirms the need for repetition of the field trial.

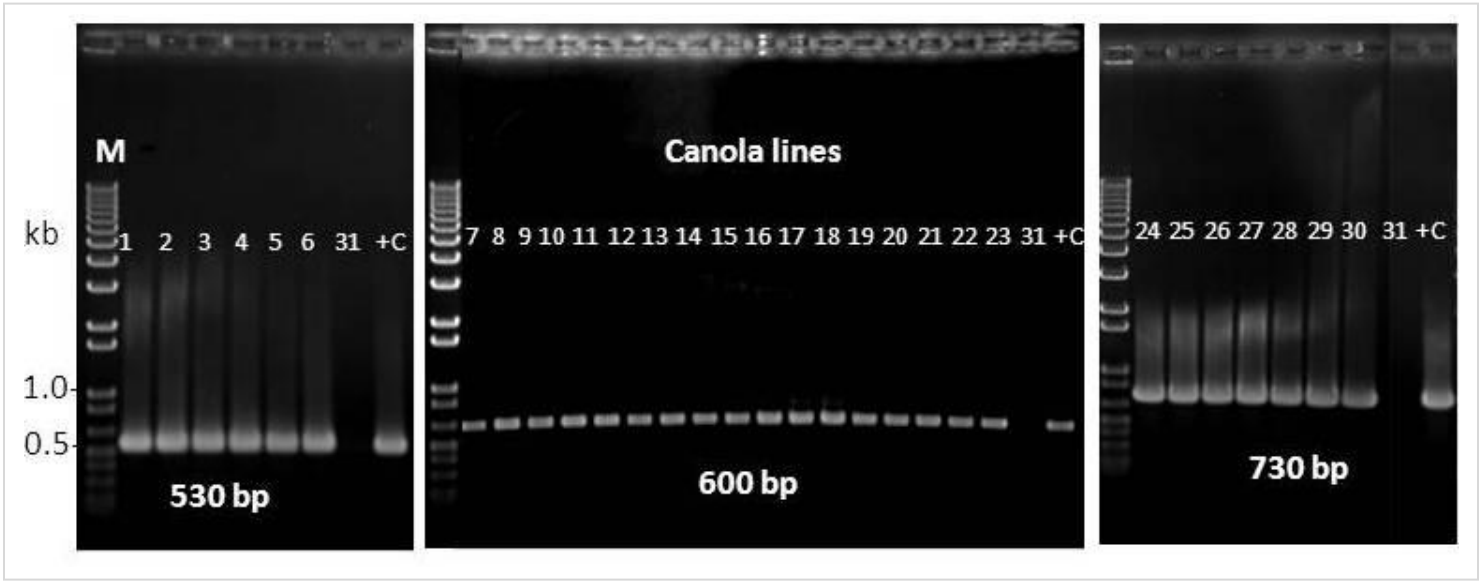


Figure 31: Genomic PCR of the canola lines tested in the field. Line 31 is non-transformed DH control. +C is PCR with recombinant plasmid DNA. Molecular weight marker (M) in kelobases (kb) is shown

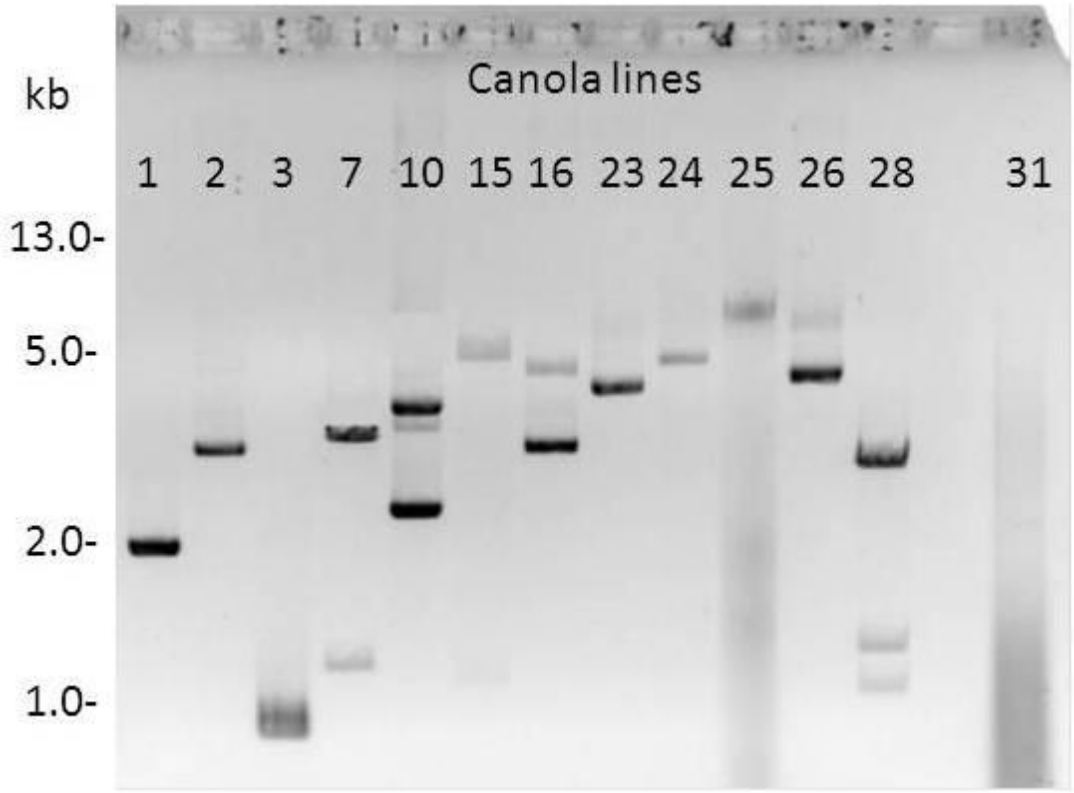


Figure 32: Southern analysis of selected canola lines. Line 31 is non-transformed DH control. Molecular weight marker in kb is shown

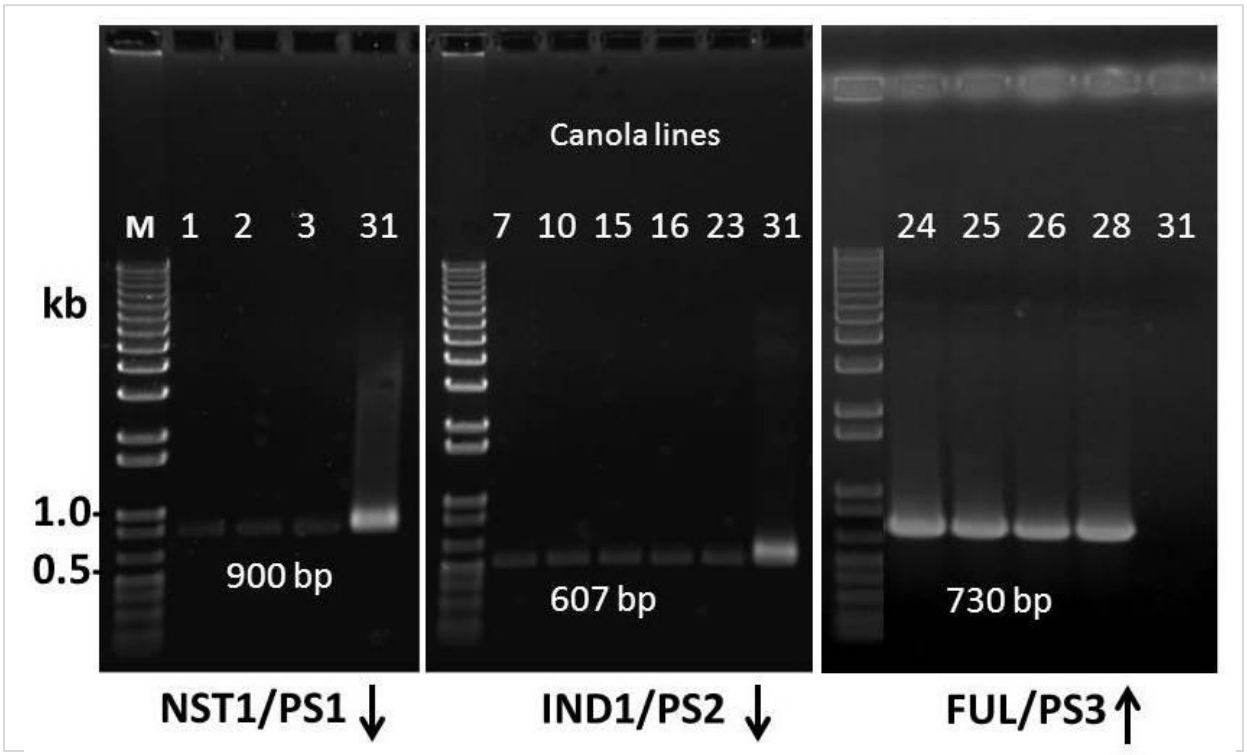


Figure 33: RT-PCR of selected canola lines. Molecular weight marker (M) in kb is shown. Intended down-regulation and up-regulation of inserted genes are shown by arrows. Line 31 is DH control

6. CONCLUSION:

The 4-year project "Developing *B. napus* lines with reduced pod shattering" was started in April 2009 and we have successfully completed the project in March 2013. Pod shattering genes (from Arabidopsis and *B. juncea*) and silique-preferred promoters were isolated, gene constructs made and canola transformation with seven constructs completed. First generation transformed canola plants were assessed for pod shattering resistance and those lines which showed reduced pod shattering and produced normal pods were grown to successive generations to confirm their shattering resistance, to develop homozygous lines and to increase seeds for field trial. Lines producing normal pods and reduced shattering (50-80% less than DH control) were tested in a field trial with CFIA authorization. Despite many challenges faced during the field trial, several lines showed reduced pod shattering in the field (30% less than DH control). These lines can be made available to canola breeders for further use in their breeding program. Through this project, we have identified homologous genes in *B. napus* which can be mutated to develop non-GMO canola with reduced pod shattering.

7. MATERIALS AND METHODS:

Chemicals and reagents

The chemicals and medium used were of: a) Chromatography grade solvents from Fisher Scientific (Whitby, ON); b) ACS grade from BDH Chemicals Inc, Toronto, ON; c) molecular biology or electrophoresis grade from Fisher BioTech, Fair Lawn, NJ; d) tissue culture grade from Life Technologies, Grand Island, NY; and e) cell culture grade from Sigma-Aldrich Canada Ltd., Oakville, ON. High performance liquid chromatography grade solvents were obtained from BDH Chemicals and EM Science, Darmstadt, Germany. Fatty Acid Methyl Esters (FAME) standards were from Nu-Chek Prep Inc., Elysian, MN. Yeast extract, agar granules, bacto-peptone and tryptone were from Difco Laboratories, Detroit, MI. Chemicals for electrophoresis were obtained from Bio-Rad Laboratories, Hercules, CA. Restriction and ligation enzymes and corresponding buffers were obtained from New England BioLabs (Beverly, MA) or Gibco-BRL (Grand Island, N.Y.) and used according to the manufacturer's instructions.

DNA extraction from gel

After the restricted DNA or PCR products were run on agarose gel, the desired DNA fragment(s) were cut out under UV light with a sharp scalpel blade and placed in a microfuge tube. The DNA was extracted from the gel piece using QIAquick gel extraction kit (Qiagen Canada Inc. Mississauga, ON). The excised gel was dissolved in 3 volumes of gel extraction buffer supplied in the kit at 50°C for 10 minutes. DNA from this solution was selectively absorbed in silica gel in QIAquick columns and eluted in 10 mM Tris-HCl, pH 8.5 or in ddH_2O by centrifugation at 10,000 g for 1 minute.

Plasmid Isolation and PCR

All plasmid isolations from DH5α and GV3101 were conducted using the QIAprep Spin Miniprep Kit (Qiagen Inc., Mississauga, Ontario, Canada) and the protocol "Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge" as outlined in the QIAprep Miniprep Handbook (Qiagen Inc., November 2005). The only difference was when isolating plasmids from GV3101, the volumes used of buffers P1, P2, and N3 were doubled.

For PCR, a master-mix was prepared containing 1X PCR Buffer (Invitrogen Inc.), 3.5mg/ml $MgCl_2$, 0.4mmol (each) dNTPs, 0.4pmol forward primer, 0.4pmol reverse primer, 0.4% Tween20, and 1.25U Taq polymerase. For the colony PCRs, the bacterial cells from the colonies were added directly into the PCR tube and for the plasmid PCRs, 1μl of purified plasmid was added to the PCR tube. The PCR tubes contained 25μl master-mix. The program run was 94^{o}C for 10 minutes, then 30 cycles of [94^{o}C for 2 minutes, 58^{o}C for 1 minute, 72^{o}C for 1 minute] followed by 72^{o}C for 10 minutes and a 4^{o}C hold.

DNA sequencing

Plasmid DNA was sequenced at Plant Biotechnology Institute, Saskatoon. The DNA sequence data were analyzed using various on-line software programs available in NCBI (http://www.ncbi.nlm.nih.gov/).

Canola transformation:

The transformation was carried out according to the method described by Moloney et al. (1989) with some modifications.

Cotyledons were excised from 6 day old seedlings grown in vitro and were dipped into an overnight suspension of PS1 or PS2 carrying *Agrobacterium* that was centrifuged and re-suspended in liquid AB medium. The cotyledon explants were then cultured on callus inducing medium [CIM: 4.6 g L^{-1} MMO (Murashige Minimal Organics), 30 g L^{-1} sucrose, 7 g L^{-1} phytagar and 4.5 mg L^{-1} benzyl adenine (BA) at pH 5.8]. After 2 days on CIM plates, the explants were transferred for 3 weeks to callus selection medium (CSM: CIM supplemented with 300 mg L^{-1} timentin and 20 mg L^{-1} kanamycin) for selection of transformed cells. For shoot development, the calluses were transferred to shoot development medium (SDM: CSM without BA). After a further 2-3 weeks, the developing shoots were transferred to root inducing medium [RIM: SDM supplemented with 0.1mg L^{-1} napthalene acetic acid (NAA)] for root formation. Shoots were maintained in this medium until rooting (normally 2-3 weeks) and then the rooted shoots were transferred into soil in the greenhouse. We have produced more than 225 transformed shoots which went through tissue culture medium before going to soil (Fig. 17).

Plant Growth

Stable transformants and control plants (DH12075) were grown in greenhouses at Alberta Innovates-Technology Futures (AITF, Vegreville, AB) in individual 15 cm diameter pots containing Sunshine Professional Growing Mix #1 (SunGrow Horticulture Canada Ltd., Seba Beach, AB). The day and night temperatures were set at 20°C and 15°C, respectively, with a daytime peak of approximately 25°C during the late spring months. Plants were not grown in the summer (May-September). The greenhouses were set on a 16h photoperiod, during which the light intensity was approximately 500 μmoles.m^{-2}.s^{-1}. Plants were fertilized on a weekly basis for 3 weeks with 10:52:10 NPK for root growth, then 20:20:20 NPK until flowering started for vegetative growth and finally with 15:11:29 NPK to encourage fruit set.

Embryo Assays

An embryo assay was done on the developing seeds of the canola plants to estimate copy number of the transgene from the segregation ratio. In this procedure, the embryos were removed from developing seeds and chopped into pieces. These pieces were put on an MS plate containing sucrose (10g/L), BA (4.5μg/ml), and kan50 for one week. Embryos containing the transgene cassette remained green, while non-transformed embryos turned white. The ratio of green to

white embryos was used to estimate transgene copy number (either single or multiple) in each plant line according to Mendelian laws.

Genomic DNA isolation and Southern blotting

Transgenic plants were verified by southern blotting (Southern 1975; Sambrook et al. 1989). DNA was isolated from 2 gm of young leaves using the Genomic DNA purification kit (Fermentas International INC, Burlington, Canada) following the manufacturer's instructions. DNA quantification was carried out using the Nano-Drop ND-1000 spectrophotometer and all samples were diluted to 1 ug/uL.

The DNA samples were digested overnight with *Hin*d III, separated on agarose gel and transferred to a Hybond N + membrane (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. DNA probes were amplified from PS1, PS2 or PS3 constructs with gene specific primers and labeled with a PCR DIG labeling kit (DIG probe synthesis Kit, Roche Diagnostics GmbH, Mannheim, Germany).The membranes were hybridized to the labeled probes and exposed to X-ray film for 1 hour before developing.

Total RNA Extraction

Total RNA was extracted from each sample, and then reverse transcribed to cDNA samples. Total RNA was prepared using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNase-free DNase set (Qiagen) was used to eliminate any genomic DNA contamination from the RNA samples. RNA concentration was measured with a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop, Wilmington, DE, USA). Absorbance ratio 260/280 nm of all RNA samples was between 2.0 and 2.2. All samples were further tested by agarose gel electrophoresis to assess the quality and integrity of the RNA.

Isolation of cDNAs:

Developing siliques of Arabidopsis ecotype Columbia were collected, flash frozen in liquid nitrogen and stored at -80^{O}C. Total RNA was extracted from 100 mg developing siliques using the Qiagen RNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada). First strand cDNA was synthesized from total RNA using the SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, USA) for amplification of cDNAs coding for transcription factors (FUL, NST1, IND).

Reverse Transcription (RT)-PCR Analysis

Total RNA was isolated from 100mg of canola developing pods (seeds removed from pods) using the RNeasy® Plant Mini Kit (Qiagen Inc.) and following the "Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi" protocol provided in the RNeasy® Mini Handbook (Qiagen Inc., April 2006).

The RT-PCR on total RNA samples was performed using the OneStep RT-PCR Kit (Qiagen Inc.) and following the "Protocol Using QIAGEN OneStep RT-PCR Kit" outlined in the OneStep RT-PCR Kit Handbook (Qiagen Inc., May 2002). In brief, first strand cDNA synthesis from mRNA was performed by a reverse transcriptase. The resulting single-strand cDNA products were amplified by PCR to produce double-stranded DNA products of the transgene. The RT step was done at 50°C for 30 minutes and the subsequent PCR program was 95°C for 15 minutes followed by 30 cycles of [94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes], then 72°C for 10 minutes and finally a hold at 4°C.

Seed oil content

Total lipid of mature seed was analyzed by low resolution-nuclear magnetic resonance (LR-NMR). Approximately 5 g mature seed were added to flat-bottomed 16 x 150 mm test tubes (to a fill height of 4 cm) and the seed oil content was measured in the Minispec LR-NMR (Bruker Optics Canada, Milton, ON). The instrument was calibrated with mature *B. napus* seed of known oil content (44-52%) obtained from Dr. James Daun of the Grain Research Laboratory of the Canadian Grain Commission (Winnipeg, Manitoba).

Lipid extraction and fatty acid (FA) analysis:

FA composition of total acyl lipid from mature seeds was determined following the International Organization for Standardization method reference number ISO 5508:1990 (E), "Animal and vegetable fats and oils—Analysis by gas chromatography of methyl esters of fatty acids". Seed lipids extracted in petroleum ether were methylated with 2% sodium methoxide in methanol for 30 minutes at room temperature and 1 μL was injected onto an Hewlett-Packard Innowax column (HP19091N) using an HP6890 gas chromatography workstation. The FA methyl esters were separated on a temperature gradient from 160°C to 180°C (20°C per minute) then 180°C to 230°C (5°C per minute) with a total run time of 37 minute per sample.

Field trail:

After obtaining authorization from CFIA (Canadian Food Inspection Agency), we conducted a confined research field test of promising lines (which showed reduced pod shattering in lab tests), along with non-transformed DH12075 control, in AITF's field at Vegreville, AB. There were a total of 31 canola lines grown in a randomized complete block design with four replications (Fig. 34). In each replication, each canola line was grown in a four-row six meter long plot (Fig. 35). Distance between canola rows in a plot was 20 cm, between plots 80 cm and between replications was 6 meters. Seeding was done on 21 May 2012, and the plots were harvested with a straight cut combine on 16th and 17th October 2012. Before harvesting, we induced pod shattering by shaking plants for fixed time with a long wood stick. Fallen seeds and fruits (due to natural wind plus shattering induction and from straight cut combining) were collected in 16 cm x 56.6 cm trays placed between canola rows; four trays per plot (Fig. 36 and 37). Harvested seeds were collected separately from each plot. From the ratio of amount of seed loss (before straight cut combine plus during straight cut combine) with the total yield per plot, we calculated pod shattering percent for each canola line.

Confined Field Trial: PS2012-**Vegreville**
Trial site area: 53.6m*42m= 2251.2 square meter
Total Area including Pollination Border: 77.6m*66m= 5121.6 square meter, or 0.52 ha

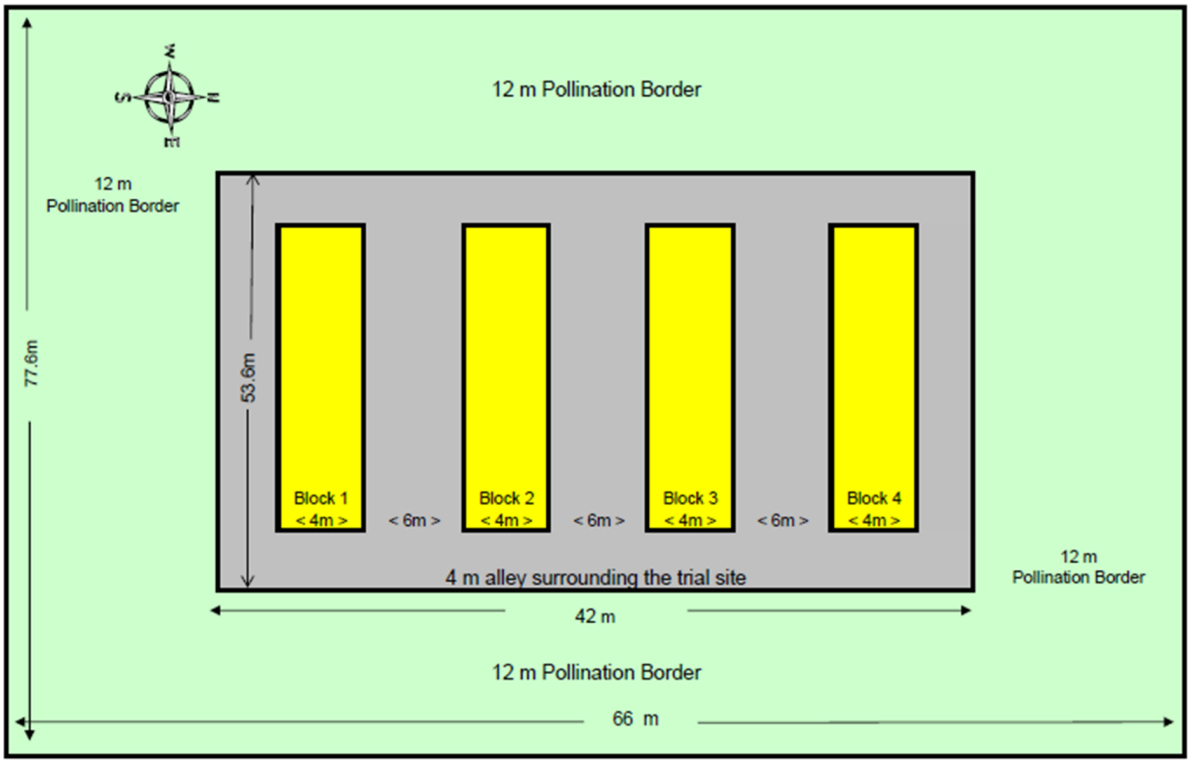


Figure 34: Field plan for 'Confined Research Field Trial' for 'Pod Shattering resistant canola' at Vegreville

Figure 35: Entire field (top panel) and individual plots (bottom panel) in the CFIA authorized field trial

Figure 36: Trays were placed between rows to collect seeds and pods

Figure 37: Straight cut combine harvesting of the field

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