Pyramiding of cry toxins and methanol producing genes to increase insect resistance in cotton

Abdul Razzaq*1,2†, Arfan Ali3†, Muhammad Mubashar Zafar†1 Xiaoying Deng1, Li Pengtao4, Ge Qun1, Muhammad Ashraf5, Maozhi Ren1, Wankui Gong1, Yuan Youlu†*

1State Key Laboratory of Cotton Biology, Key Laboratory of Biological and Genetic Breeding of Cotton, The Ministry of Agriculture, Institute of Cotton Research, Chinese Academy of Agricultural Science, Anyang, 455000, Henan, China.
2Institute of Molecular Biology and Biotechnology, The University of Lahore, Lahore-Pakistan.
3FB Genetics, Four Brothers Group, Lahore-Pakistan.
4School of Biotechnology and Food Engineering, Anyang Institute of Technology, Anyang 455000, Henan, China.
5University of Agriculture Faisalabad, Faisalabad-Pakistan.
† Joint first Authors, contributed equally

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*Corresponding Authors:
Yuan Youlu, Email: yuanyoulu@caas.cn
Abdul Razzaq, Email: biolfomanite@gmail.com
Abstract:

The idea of enhanced methanol production from cell wall by pectin methyl esterase enzymes (PME) combined with expression of cry genes from Bacillus thuringiensis as a strategy to improve pests control in cotton is presented. We constructed a cassette containing two cry genes (cry1Fa and Cry32Aa) and two pme genes, one from Arabidopsis thaliana (AtPME), and other from Aspergillus niger (AnPME) in pCAMBIA1301 plant expression vector using CAMV-35S promoter. This construction was transformed in Eagle-2 cotton variety using shoot apex-cut Agrobacterium-mediated transformation. The expression of cry genes and pme genes was confirmed by qPCR. Methanol production was measured in control and in the cry and pme transformed plants showing methanol production only in transformed plants, then the non-transgenic cotton plants. Finally, insect bioassays performed with transgenic plants expressing cry and pme genes, showed 100% mortality for Helicoverpa armigera (cotton bollworm) larvae, 70% mortality for pectinophore gossypiella (pink bollworm) larvae and 95% mortality of Earias fabia, (spotted bollworm) larvae, that was higher than the transgenic plants expressing only cry genes that showed 84%, 49% and 79% mortality, respectively. These results demonstrate that Bt. cry-genes coupled with pme genes is an effective strategy to improve the control of different insect pests.

Key Words: Cotton, Transformation, Eagle-2, Agrobacterium, pectin methyl esterase enzyme, insecticidal Cry proteins.
1. Introduction

_Gossypium hirsutum_ L. is an important economical crop and one of the largest sources of natural fiber worldwide. Cotton crop is subjected to various biotic and abiotic stresses throughout its life cycle. However, the biotic stress caused by pathogens and pests has an important negative impact not only on yield and quality, but also in control measures that increases production cost globally. Cotton fields are prone for lepidopteran infestation such as pink bollworm (_Pectinophora gossypiella_), army bollworm (_Spodoptera litura_), American bollworm (_Heliothis armigera_) and spotted bollworm (_Earias fabia_). The use of chemical insecticides is not an adequate solution since they seriously damage the environment and human health. _Bacillus thuringiensis_ (Bt) are soil bacteria, that produce different insecticidal proteins (named δ-endotoxins, such as Cry toxins), which have been successfully used against insect pests attack and several Cry insecticidal proteins have been also transformed into cotton crops since 1996. The effectiveness of Bt δ-endotoxins started to decline due to the development of resistance by the insect pests. Despite of the proven substantial effects of transgenic Bt-cotton plants against insect attack, still is needed to improve this technology, for instance by combining with some enzymes involved in defense strategies against insect pests. The overproduction of enzymes, involved in insect defense, can be a good alternative to reduce the pest attack and development of insect resistance to Cry toxins.

The plant cell walls are heterogeneous structures containing cellulose, hemicellulose, pectin, phenolic compounds and cell wall proteins. Pectins are integral components of plant primary cell wall acting as barriers to insect pests. Pectin methyl esterase enzyme (PME) catalyzes dersterification of pectin into pectate and methanol in the plant cell wall to regulate an inhibitory response against insect pests. Multiple mechanisms are involved in regulation of PME activity and methanol production in plants such as cell wall pH modifications, expression of inhibitory proteins and differential isoforms expression in different tissues at different stages.

In this work, PME from _Arabidopsis thaliana_ (AtPME, accession no. NP 566842) and _Aspergillus niger_ (AnPME, accession no. XM_001390469) were used for their overexpression coupled with insecticidal cry genes in transgenic cotton and toxicity was evaluated against different Lepidopteran insect pests. We selected to work with the PME
isolated from the fungus *A. niger* shows high methanol production in tobacco cell suspension culture. It is also reported that activity of PME is elicited by the attack of herbivores in different plant species, inducing the production of toxic methanol. Methanol is toxic to insects pests, damaged and wounded leaves by the insect attack as a primary source of methanol production by the pre-existing PME in the cell wall.

2. Results

2.1 Detection of pme or cry genes cassettes in pUC57 vector through restriction digestion

The two plasmids pUC57 that we received from BioBasic, were digested with *BamHI* and *EcoRI* or with *EcoRI* and *HindIII* and resolved on 0.8% agarose gel as shown in fig.1. The expected sizes of 7954 bp and 7893 bp respectively for the cassettes containing *cry* genes or *pme* genes cassettes were observed (Fig. 1). A schematic representation of both gene cassettes containing *cry1Fa* and *cry32Aa* genes or *AtPME* and *AnPME* genes, respectively is also shown in this figure.
Figure 1: Restriction analysis of puc57 vectors containing the cry1Fa and cry32Aa gene cassette (total 7954 bp size) or the AtPME and AnPME gene cassette (total 7893 bp size). Panel A, BamHI and EcoRI restriction analysis of the cry1Fa and cry32Aa gene cassette. M: Lane 1, Kb ladder. Lane 2, cry1Fa and cry32Aa gene cassette positive sample. Lanes 1, negative control of pUC57 without insert samples. Panel B, EcoRI and HindIII restriction analysis of the AtPME and AnPME gene cassette. M: 1 Kb ladder. Lanes 1, 2 AtPME and AnPME gene cassette positive samples. Panel C, schematic representation of both gene cassettes containing cry1Fa and cry32Aa genes or AtPME and AnPME genes, respectively.

2.2 Detection of transgene in pCAMBIA through restriction digestion analysis and PCR

The purified DNA bands containing these two gene cassettes were ligated to the plant expression vector pCAMBIA1301 pre-digested with the corresponding restriction enzymes. The resulting plasmids were confirmed by restriction digestion analysis. Digestion with EcoRI and HindIII was done for the plasmid containing cry genes cassette and digestion with EcoRI and BamHI was done for the plasmid containing pme genes cassette. The observed band sizes, confirmed their successful ligation in pCAMBIA1301. Both cry genes were then amplified resulting in a PCR product of 459 bp for cry1F and 462 bp for cry32A. Similarly, AnPME and AtPME genes were amplified through colony PCR resulting in a PCR product of 557 bp and 554 bp respectively (Fig 2).
Figure 2. Restriction digestion and PCR analyses of both *cry* genes and *pme* genes cassettes.

Panel A, *EcoRI* and *BamHI* restriction of pCAMBIA1301 vector containing *cry* genes cassette; lanes 1, 2 show positive samples; Panel B, *EcoRI* and *HindIII* restriction of pCAMBIA1301 vector containing *pme* genes cassette; lanes 1, 2 show positive samples; Panel C, confirmation of *cry1Fa* gene by PCR, M: 1 Kb ladder; Lane 1, shows negative control; Lane 3 shows positive control; Lanes 4, 5, 7, 8, 11 and 12, show positive samples; Lanes 6, 9, and 10, show negative samples. Panel D, confirmation of *cry32Aa* gene by PCR, M: 1Kb ladder; Lane 1, 3, shows negative control; Lanes 4, 5, 7, and 8 shows positive samples; Lanes 6, 9, and 10 show negative samples. Panel E, confirmation of *AnPME* gene by PCR, M: 1 Kb ladder; Lane 1, shows positive control; Lanes 3, 4, and 6, show positive samples; Lanes 2, and 5, show negative samples. Panel F, confirmation of *AtPME* gene by PCR, M: 1 Kb ladder; Lane 1, shows positive control; Lanes 3, 4, 5, and 6, show positive samples; Lane 2, shows a negative sample.

2.3 Detection of pCAMBIA1301-PMECassette and pCAMBIA1301-cryCassette constructions transformed into A. *tumefaciens*

A number of random colonies of electroporated *A. tumefaciens* strain LBA4404 were selected for PCR colony assays, using specific primers designed from *cry1Fa, cry32Aa, AnPME* and *AtPME* genes. The PCR products were resolved on 1.5% agarose gel and our results show that all the colonies amplified the expected PCR product of 459 bp and 462 bp for the *cry1F* and *cry32A* respectively and 557 bp and 554 bp for the *Anpme* and *Apme* genes respectively, with the exception of the negative control (Fig. 3).

Figure 3: PCR amplification of both *cry* genes and *pme* genes from transformed *A. tumefaciens* with the corresponding pCAMBIA1301-*cryCassette* and pCAMBIA1301-*pmeCassette* constructions.

Panel A, Amplification of both *cry* genes; M: 1 kb ladder; Lane 1, shows negative control; Lane 2, shows positive control; Lanes 3-6 show positive amplification of *cry1Fa*, and Lane 7-10 show positive
amplification of cry32Aa genes in individual A. tumefaciens colonies. Panel B, shows an amplification of both pme genes, M: 1 Kb ladder; Lane 1, shows positive control; Lane 2, shows negative control; Lanes 3, 4 show positive amplification of AnPME, and Lane 5, 6 show positive amplification of AtPME genes in individual A. tumefaciens colonies.

2.4 Transformation of the two double gene cassettes into Cotton plants

The seeds of Gossypium hirsutum Eagle-2 were sterilized and placed in the dark at 30 °C for 48 h. The germinated seedlings were used for transformation using shoot apex cut method as shown in figure 4. These plants were inoculated with both A. tumefaciens strains containing cry genes cassette or pme gene cassettes. Germination index of Eagle-2 was calculated to be 67.50% as shown in tables 1 and 2. Similarly, transformation efficiency was recorded to be 0.49% (table 3).
Figure 4: transformation procedure of cry and PME genes in Eagle-2 cotton plants. Panel A and B, soaking of seeds; Panel C and D, shifting of embryos on MS plates; Panel E, F and G shifting of embryos into the MS tubes; Panel H, I and J, Shifting of plants into the pots.

2.5 Detection of transgenic cotton plants

Fresh leave samples of the transgenic cotton plants were selected for DNA isolation and analysis of successfully transformed cry genes and pme genes using specific primers that produce PCR products of 459 bp and 462 bp for cry (cry1F & cry32A) genes whereas 557 bp and 454 bp for pme (Anpme & Atpme) genes, respectively (Fig. 5).

Figure 5: Determination of cry genes and PME genes expression in transgenic cotton plant by PCR analysis.

Panel A, M: 1 Kb ladder; Lane 1, 2 shows negative sample; Lanes 3, 4 show positive for cry1Fa and Lane 5, 6 show positive for cry32Aa, positive samples. Panel B, M: 1 Kb ladder; Lane 1, shows positive control; Lane 2, shows negative control; Lanes 3, 5, show positive for AnPME, and Lane 6, 7 show positive for AtPME samples; lane 4, shows negative sample.

The mRNA from transgenic plants was isolated and cDNA transcribed. The relative mRNA expression of cry1Fa, cry32Aa, AtPME and AnPME genes was analyzed by using SYBR Green Mix in qPCR assays and higher expression of these genes was found in transgenic plants (Fig. 6). Expression of GAPDH gene was used as internal control reference for normalization in these assays. We analyzed the expression of these genes in four transgenic plants (named K1, K2, K3 and K4) that were positive for transformation with the four genes (cry1Fa, cry32A, AtPMe and AnPME). Control plants were non-transformed Eagle-2 cotton plants. The K2 plants showed the highest relative highest expression of the four AtPME and AnPME, cry1Fa and cry32A genes. While K3 plants showed the lower expression of these genes.
Figure 6: Relative expression of *cry* and *PME* genes in four transgenic cotton plants (K1, K2, K3 and K4). The relative expression of *cry* and *PME* analyzed in different plants shown in the figure was calculated according to the $2^{\Delta\Delta Ct}$ method using GAPDH as internal control reference gene for normalization.

### 2.6 Evaluation of methanol concentration in transgenic cotton plants

Transgenic K1-K4 cotton plants were subjected to Mass Spectrometry (MS) for methanol quantification. Transgenic plants K1 and K2 showed the highest contents of methanol, respectively, as compared to K3 and K4 plants and control plants. These data indicated that the methanol production in transgenic plants showed higher values than non-transgenic cotton plants.
Figure 7: Methanol quantification in transgenic cotton plants. Standard curve of methanol was done by Mass Spectrometry (MS) with reference to a methanol standard. The transgenic cotton plants, namely, K1, K2, K3, K4 were subjected to methanol quantification.

2.7 Evaluation of transgenic cotton plants for resistance against chewing insects (*H. armigera* and *P. gossypiella*).

Bioassays were performed by using fresh leaves of non-transgenic cotton as negative control, and compared with transgenic cotton plants K1 to K4 expressing double Bt-cry genes and *pme* genes. We also compared with cotton plants transformed only with the two Bt cry-genes carried out in the laboratory. Each of the sampled fresh leaves were infected with *H. armigera* and *P. gossypiella* larvae. A 5-7 larvae of 3rd instar per leaf were used in triplicates.
The infestation *H. armigera* data were recorded, showing 100% mortality on the third day after infection of transgenic cotton plants harboring both Bt *cry*-genes coupled with *AtPME* and *AnPME*, while 84% mortality was observed after fifth days in the control plants transformed only with Bt-*cry1Fa* and *cry32* genes and no mortality was observed in negative control of Eagle-2 cotton plants as shown in figure 8.

In the case of *P. gossypiella* larvae were released on freshly growing bolls of the cotton plants. Transgenic cotton plants expressing *AtPME* and *AnPME* along with Bt *cry*-genes showed 70% mortality of *P. gossypiella* larvae after 3 days, implying resistance towards *P. gossypiella* larvae. In contrast to transgenic cotton plants harboring only the two Bt *cry*-genes that showed 49% of *P. gossypiella* mortality and 0% mortality was observed in non-transgenic Eagle-2 control plants.

Finally, regarding spotted Bollworm (*Earias fabia*) 95% mortality was observed in transgenic harboring double Bt *cry* and *pme* genes cassettes, while 79% mortality was observed in transgenic plants expression only the double Bt *cry*-genes (Fig. 9). Two-way ANOVA showed the significance of our data at $P \leq 0.0001$ (Fig. 10).

**Figure 8**: Cotton leaf chewing and mortality assay of *H. armigera* in different transgenic cotton plants.
Panel A, Non-transgenic cotton plant leaf completely chewed by H. armigera. Panel B, Double Bt-gene (cry1Fa and cry32Aa) plants showed resistance and a little bit chewed by H. armigera. Panel C, Double Bt-gene (cry1Fa and cry32Aa) and double AnPME and AtPME gene in the same plant showed full resistance and just a minor cut was observed by H. armigera. Panel D, Hatching of H. armigera on negative control Eagle-2 cotton plant leafs. Panel E, in double Bt-gene (cry1Fa and cry32Aa) cotton plant leafs, the H. armigera were dead after eating scratched looking portion of leaf. Panel F, in the double Bt-gene (cry1Fa and cry32Aa) and double AnPME and AtPME gene plants, the H. armigera were dead even after a minor first cut.

Fig 9: Pink Bollworm P. gossypiella mortality assays in different transgenic cotton plants. Panels A and B, in non-transgenic cotton plants, the cotton boll showed to be completely damaged by P. gossypiella and and larvae that were still alive are highlighted with a red circle mark. Panels C and D, in transgenic plants transformed with the double Bt-gene (cry1Fa and cry32Aa), the plant bolls showed insect resistance and only one locule was damaged but P. gossypiella larvae were dead. Panels E and F In the transgenic plants transformed with double Bt-gene (cry1Fa and cry32Aa) and double AnPME and AtPME gene in the same plant; the plants showed full resistance and even spotted was found dead on leaf and P. gossypiella free cotton was fully developed.
Figure 10: Mortality analysis against army boll worm *Helicoverpa armigera*, Pink boll worm *Pectinophora gossypiella* and spotted bollworm *Earias fabia*. Two-way ANOVA showed the significance of the data compared to the non-transformed plants.

3. Discussion

Cotton is considered a socio-economically important crop\(^\text{16-18}\) and due to current trend of global market it is necessary to make continuous improvements in cotton varieties\(^\text{19}\). The transformation of new genes into cotton varieties is required to develop resistance against unrelated invading insect pests\(^\text{20}\). For this goal, a unique approach was adopted therein two cassettes were designed. One cassette harbor two pectin methyl esterase genes (*pme*) from *A. thaliana* and *A. niger* and second cassette contains two insecticidal proteins coded by *cry* genes from *B. thuringiensis*. Both cassettes were transformed into non-transgenic cotton variety Eagle-2 to achieve resistance against insects as previously depicted in tobacco plants\(^\text{8}\). Initial screening of putative cotton plants was done by hygromycin as reported\(^\text{21}\). Putative transgenic cotton plants were confirmed through PCR by using specific primers as previously done\(^\text{22}\). PCR confirmed transgenic plants were subjected to qRT-PCR for the determination of mRNA expression of the transgenic genes as reported\(^\text{23}\). Ultimately, the efficacy of transgene was determined by insect bioassay against different target *Lepidopteran* larvae\(^\text{24}\).
Naturally, plants produce methanol, which is non-toxic for the plants \(^{25}\). Methanol after accumulation in leaves is released into the atmosphere through stomata \(^{26,27}\) and is found toxic to the insects \(^{8}\). In this study, the methanol quantification was done in transgenic and non-transgenic plants by MS as reported before \(^{10}\). Transgenic cotton plants K1 and K2 showed a 0.7% and 0.9% increase in methanol concentration when compared with the non-transgenic control plants, while K3 and K4 transformed plants showed only 0.5% and 0.4% methanol concentration, which is less than K1 and K2 plants, but greater than a control plant. Methanol concentration was calculated in correlation with insect bioassay in which K1 and K2 with higher expression of all genes showing 100% mortality in accordance with Hasunuma et al. (2003).

In this study, RT-qPCR assays were conducted to analyze mRNA expression of \textit{pme} and \textit{cry} genes in transgenic cotton plants. The relative expression of \textit{AtPME} and \textit{AnPME} was higher in K2, than in control plants. Insect bioassays were performed on detached leaves, flower and bolls of cotton. The infestation data were recorded and 100% mortality was observed after three days in transgenic cotton plants harboring both Bt \textit{cry}-genes coupled with \textit{AtPME} and \textit{AnPME} genes, while 84% mortality was observed in Bt \textit{cry}-genes transgenic cotton plants after five days, while there was no mortality observed in negative control cotton plants as shown in fig. 8. The Pink Bollworm (\textit{P. gossypiella}) larvae were released on freshly growing bolls of the cotton plants. Transgenic cotton plants showed resistance towards pink bollworm larvae for 3 days with 70% mortality in case of transgenic cotton plants harboring \textit{AtPME} and \textit{AnPME} along with Bt \textit{cry}-genes in contrast with the 49% mortality observed in transgenic cotton plants harboring only the two Bt \textit{cry}-genes and 0% mortality in non-transgenic control plants. Finally, we observed 95% mortality in spotted bollworm in transgenic plants harboring double Bt-genes and \textit{pme} genes, while only 79% mortality was observed in transgenic plants expressing only the double Bt \textit{cry}-genes as shown in Fig. 9. These results indicated that Bt \textit{cry}-genes coupled with \textit{pme} genes is a possible and useful strategy to control different insect pests and for lowering the resistance of insects against transgenic cotton varieties.
4. Materials and Methods

4.1 Plant materials

Gossypium hirsutum L. Eagle-2 variety was selected for the expression of two cry genes (cry1Fa and cry32Aa) and two methanol producing genes (AtPME and AnPME). Eagle-2 seeds were obtained from Four Brothers Seeds Multan-Pakistan and planted in a specific field from Four Brothers Lahore-Pakistan.

4.2 Sequence selection and plasmid construction

Gene sequences of selected pme genes (AtPME, accession no. NP 566842; AnPME, accession no. XM_001390469) were taken from NCBI and after codon optimization, were synthesized by BioBasic, Canada. The synthetic double gene AnPME, AtPME cassette (total 7893 bp) was cloned into the EcoRI and HindIII restriction sites of puc57 vector. While the cry1Fa, cry32Aa gene cassette (total 7954 bp) was cloned into the EcoRI and BamHI restriction sites of pUC57 vector. All genes were under regulation of CaMV35S promoter and Nos terminator was added at the end of these genes (Fig 1).

4.3 Cloning of genes into plant expression vector pCAMBIA1301

The two pUC57 vectors with the AtPME, AnPME cassette, or the cry1Fa, cry32Aa cassette were transformed into Escherichia coli top10 competent cells through heat shock method and selected on LB media supplemented with tetracycline (50 µg/ml) and ampicillin (50 µg/ml). Plasmids were isolated by using Gene Jet plasmid DNA isolation kit (Thermo Scientific, Vilnius, Lithuania) as indicated in the manufacturer protocol. The presence of these genes was confirmed through restriction digestion using EcoRI and HindIII or using EcoRI and BamHI enzymes accordingly to each plasmid. The 7.8 DNA band of AtPME, AnPME cassette and 7.9 Kb band of cry1Fa, and cry32Aa cassette were visualized in 0.8% of agarose gel electrophoresis and excised fragments were purified by using Gene JET Gel Extraction Kit (Thermo Scientific, Vilnius, Lithuania). The purified DNA bands were then ligated to the plant expression vector pCAMBIA1301 pre-digested with the corresponding restriction enzymes and transformed into E. coli top10 competent cells. A 1Kb DNA size marker (250bp-10Kb) was received from GeneRuler™ and used in this study.

4.4 Transformation of pCAMBIA1301 vectors with pme and cry genes cassettes into Agrobacterium tumefaciens.
The ligation of the two excised double genes cassettes in pCAMBIA1301 vector was confirmed by restriction digestion using EcoRI and BamHI enzymes or EcoRI and HindIII accordingly to the pCAMBIA1301-cryCassette or to the pCAMBIA1301-pmeCassette, and further confirmed by PCR using specific primers An-primers [Forward 5′-GGTGCTATCGTTGTTGCTAAGTC-3′ and reverse 3′-GCAGTAATTGAAGCAGATGAAGG] and At-primers [Forward 5′-TCTGTTCTTTGGGTAACACTTG-3′ and reverse 3′-GTGATCACGACCTAAGAAAGAC-5’]. The optimized PCR conditions used for Cry genes; Initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 45 seconds, annealing at 59°C for 50 seconds, extension at 72°C for 1:30 minutes, final extension at 72°C for 10 minutes whereas the PCR conditions for pme genes; Initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 45 seconds, annealing at 57°C for 45 seconds, extension at 72°C for 1:30 minutes, final extension at 72°C for 10 minutes. The confirmed plasmids were transformed into A. tumefaciens strain LBA4404 competent cells by electroporation. The transformant A. tumefaciens cells were grown on YEP media (Peptone 10 g/L, Yeast extract 10 g/L, Sodium chloride 5 g/L, pH 7.5) supplemented with Kanamycin (50 mg/ml) and Rifampicin (50 mg/ml). The appeared colonies of A. tumefaciens were then further evaluated through PCR in colony as reported using the specific primers.

4.5 Agrobacterium tumefaciens mediated transformation of cotton plants

The seeds of Gossypium hirsutum Eagle-2 plant were surface sterilized and placed in the dark at 30 °C for 48 h. The germinated seedlings were used for co-transformation using shoot apex cut method. Embryos, after injury, were inoculated with the two selected transformant A. tumefaciens strains harboring both double gene constructs in MS medium (4.4 g/L, Sucrose 30 g/L, Phytagel 2.4 g/L) cultured for 1 h at 28°C. The embryos were allowed to grow on MS medium plates supplemented with cefotaxime (500 mg/ml) followed by screening in MS tubes supplemented with hygromycin (25 mg/ml) for one and a half month. After screening, the cotton plants from the tubes were shifted into pots containing equal proportion of clay, peat moss and sand (1:1:1). Subsequently, the putative transgenic cotton plants were shifted to the greenhouses of Four Brothers Genetics Inc. for acclimatization and hardening followed by molecular analysis.
4.6 Detection of the two double gene cassettes in cotton plants through PCR
Leaves of the putative transgenic cotton plants were taken for the confirmation of both
double genes AnPME, AtPME and cry1Fa, cry32Aa cassettes, through PCR using
manufacturer protocol Green Plant direct PCR master mix kit, (Thermo Scientific) using
specific primers. In addition, virG gene amplification was also done, by using a specific
set of primers from the vir region, to nullify the Agrobacterium contamination. The PCR
annealing temperature was set at 60°C.
4.7 RNA extraction and cDNA preparation
RNA from putative cotton plants was isolated using Agilent kit (Agilent Technologies,
Santa Clara, USA). The RNA was quantified in ng/µl using NanoDrop ND-1000
spectrophotometer at 260 and 280 nm. The DNase-treated total RNA was used to prepare
cDNA using the first strand cDNA synthesis kit (Thermo Scientific) and cDNA was
stored at -20 °C.
4.8 Expression analysis of cotton transgene
Expression analysis of transgenes was performed by qPCR using specific primers in
triplicates with a product size of < 200 bp following the protocol of Maxima SYBR
Green/ROX (Thermo Scientific). The reaction mixture was prepared in a total of 20 µl
with the following components of 1 µl of 10 pmol of forward and reverse primers, 5 µl of
Maxima® SYBR Green/ROX qPCR Master Mix (2x) and 1 µl (50 ng/µl) of cDNA.
Sequences of the primers used for the amplifications of both the genes are given in table
1. Relative expression was determined according to the $2^{(-\Delta\Delta Ct)}$ method using GAPDH
primers were used as internal control reference gene for normalization in these qPCR
experiments. All assays were done in triplicate.
4.9 Methanol quantification in transgenic cotton plants
Transgenic cotton leaves (1g) were used for determining methanol concentration.
Phosphate buffer was prepared in deuterium oxide composed of 0.03% (w/v) sodium salt
of trimethylsilyl propionic acid (TSP) (Sigma-Aldrich). After sonication of the samples,
they were centrifuged at 13,000 ×g for 10 min and supernatant was collected in a tube for
methanol content determination, by using mass spectrometry (MS) with the procedure
described by 10. Different concentrations of methanol from 0 to 20% were used as
standards.
4.10 Insect bioassays

The efficacy of methanol overproduction was tested on insect bioassays against *Helicoverpa armigera, Pectinophora gossypiella* and *Earias fabia* larvae by comparing toxicity of non-transgenic and transgenic cotton plants. The upper positive leaves of the plant that have *H. armigera* and *E. fabia* larvae were removed and placed on moist filter paper in laboratory conditions. A 5-7 larvae of 3rd instar were used per leaf in triplicate.

The efficacy of transgenic plants against pink bollworm (*P. gossypiella*) was evaluated by releasing the larvae on young growing bolls and flowers in the field. The mortality rate was observed continuously for 7 days. The mortality rate was calculated by the following formulae;

\[
\% \text{ Mortality} = \frac{\text{No of dead Larvae}}{\text{Total No. of Larvae}} \times 100
\]

5. Conclusion

Transgenic plants expressing simultaneously *pme* and *cry* genes were evaluated against different Lepidopteran insect pests and compared against non-transgenic and transgenic plants expressing only Bt *cry*-genes. Increased mortality in insects was observed in transgenic plants harboring *pme* and *cry* genes combination as compared with positive control expressing *cry* genes only. The increased production of ethanol by *pme* genes in these transgenic plants explains their improvement in the control against insect attack. This control strategy infers that it may be robust to reduce the attack of different lethal cotton insects in order to maintain the plant health and to increase the yield. As it is reported that insect attack has become a major concern in cotton growing countries around the world especially in Pakistan where farmers have started walking out from the cultivation of cotton due to the high risk of insect attack leading to the poor yield which in uneconomical for the farmers. Therefore, the proposed strategy may incur some positive results to win the farmer’s interest in the cotton cultivation.
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7. Ethics approval and consent to participate

Not applicable.

8. Consent for publication

Not applicable.

9. Availability of data and material

Not applicable.

10. Conflict of interest

Authors declare that they have no conflict of interest for the publication of the manuscript.

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12. Authors' contributions
AR and AA wrote the initial draft of the manuscript and equal are contributor. MMZ, DX MA and LP made all necessary corrections and carried out final editing of manuscript. MR, WG and GQ proof read the manuscript. Final approval for publication was given by the group leader at institute of cotton research YY.

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Figure legends

**Figure 1:** Restriction analysis of puc57 vectors containing the *cry1Fa* and *cry32Aa* gene cassette (total 7954 bp size) or the *AtPME* and *AnPME* gene cassette (total 7893 bp size).

Panel A, *BamHI* and *EcoRI* restriction analysis of the *cry1Fa* and *cry32Aa* gene cassette. M: Lane 1, Kb ladder. Lane 2, *cry1Fa* and *cry32Aa* gene cassette positive sample. Lanes 1, negative control of pUC57 without insert samples. Panel B, *EcoRI* and *HindIII* restriction analysis of the *AtPME* and *AnPME* gene cassette. M: 1 Kb ladder. Lanes 1, 2 *AtPME* and *AnPME* gene cassette positive samples. Panel C, schematic representation of both gene cassettes containing *cry1Fa* and *cry32Aa* genes or *AtPME* and *AnPME* genes, respectively.

**Figure 2.** Restriction digestion and PCR analyses of both *cry* genes and *pme* genes cassettes.

Panel A, *EcoRI* and *BamHI* restriction of pCAMBIA1301 vector containing *cry* genes cassette; lanes 1, 2 show positive samples; Panel B, *EcoRI* and *HindIII* restriction of pCAMBIA1301 vector containing *pme* genes cassette; lanes 1, 2 show positive samples; Panel C, confirmation of *cry1Fa* gene by PCR, M: 1 Kb ladder; Lane 1, shows negative control; Lane 3 shows positive control; Lanes 4, 5, 7, 8, 11 and 12, show positive samples; Lanes 6, 9, and 10, show negative samples. Panel D, confirmation of *cry32Aa* gene by PCR, M: 1Kb ladder; Lane 1, 3, shows negative control; Lanes 4, 5, 7, and 8 shows positive samples; Lanes 6, 9, and 10 show negative samples. Panel E, confirmation of *AnPME* gene by PCR, M: 1 Kb ladder; Lane 1, shows positive control; Lanes 3, 4, and 6, show positive samples; Lanes 2, and 5, show negative samples. Panel F, confirmation of *AtPME* gene by PCR, M: 1 Kb ladder; Lane 1, shows positive control; Lanes 3, 4, 5, and 6, show positive samples; Lane 2, shows a negative sample.

**Figure 3:** PCR amplification of both *cry* genes and *pme* genes from transformed *A. tumefaciens* with the corresponding pCAMBIA1301-*cryCassette* and pCAMBIA1301-*pmeCassette* constructions.

Panel A, Amplification of both *cry* genes; M: 1 kb ladder; Lane 1, shows negative control; Lane 2, shows positive control; Lanes 3-6 show positive amplification of *cry1Fa*, and
Lane 7-10 show positive amplification of cry32Aa genes in individual A. tumefaciens colonies. Panel B, shows an amplification of both pme genes, M: 1 Kb ladder; Lane 1, shows positive control; Lane 2, shows negative control; Lanes 3, 4 show positive amplification of AnPME, and Lane 5, 6 show positive amplification of AtPME genes in individual A. tumefaciens colonies.

**Figure 4:** transformation procedure of cry and PME genes in Eagle-2 cotton plants. Panel A and B, soaking of seeds; Panel C and D, shifting of embryos on MS plates; Panel E, F and G shifting of embryos into the MS tubes; Panel H, I and J, Shifting of plants into the pots.

**Figure 5:** Determination of cry genes and PME genes expression in transgenic cotton plant by PCR analysis.

Panel A, M: 1 Kb ladder; Lane 1, 2 shows negative sample; Lanes 3, 4 show positive for cry1Fa and Lane 5, 6 show positive for cry32Aa, positive samples. Panel B, M: 1 Kb ladder; Lane 1, shows positive control; Lane 2, shows negative control; Lanes 3, 5, show positive for AnPME, and Lane 6, 7 show positive for AtPME samples; lane 4, shows negative sample.

**Figure 6:** Relative expression of cry and PME genes in four transgenic cotton plants (K1, K2, K3 and K4).

The relative expression of cry and PME analyzed in different plants shown in the figure was calculate according to the $2^{-\Delta\Delta Ct}$ method using GAPDH as internal control reference gene for normalization.

**Figure 7:** Methanol quantification in transgenic cotton plants. Standard curve of methanol was done by Mass Spectrometry (MS) with reference to a methanol standard. The transgenic cotton plants, namely, K1, K2, K3, K4 were subjected to methanol quantification.

**Figure 8:** Cotton leaf chewing and mortality assay of H. armigera in different transgenic cotton plants.

**Panel A,** Non-transgenic cotton plant leaf completely chewed by H. armigera. **Panel B,** Double Bt-gene (cry1Fa and cry32Aa) plants showed resistance and a little bit chewed by H. armigera. **Panel C,** Double Bt-gene (cry1Fa and cry32Aa) and double AnPME and
AtPME gene in the same plant showed full resistance and just a minor cut was observed by *H. armigera*. **Panel D**, Hatching of *H. armigera* on negative control Eagle-2 cotton plant leafs. **Panel E**, in double Bt-gene (*cry1Fa* and *cry32Aa*) cotton plant leafs, the *H. armigera* were dead after eating scratched looking portion of leaf. **Panel F**, In the double Bt-gene (*cry1Fa* and *cry32Aa*) and double *AnPME* and *AtPME* gene plants, the *H. armigera* were dead even after a minor first cut.

**Fig 9**: Pink Bollworm *P. gossypiella* mortality assays in different transgenic cotton plants. **Panels A and B**, in non-transgenic cotton plants, the cotton boll showed to be completely damaged by *P. gossypiella* and and larvae that were still alive are highlighted with a red circle mark. **Panels C and D**, in transgenic plants transformed with the double Bt-gene (*cry1Fa* and *cry32Aa*), the plant bolls showed insect resistance and only one locule was damaged but *P. gossypiella* larvae were dead. **Panels E and F** In the transgenic plants transformed with double Bt-gene (*cry1Fa* and *cry32Aa*) and double *AnPME* and *AtPME* gene in the same plant; the plants showed full resistance and even spotted was found dead on leaf and *P. gossypiella* free cotton was fully developed.

**Figure 10**: Mortality analysis against army boll worm *Helicoverpa armigera*, Pink boll worm *Pectinophora gossypiella* and spotted bollworm *Earias fabia*. Two-way ANOVA showed the significance of the data, compared to the non-transformed plants.
Table 1: Germination Index

| No. of petri plates | Total seeds | No. of germinated seeds | No. of ungerminated seeds | Germination index |
|---------------------|-------------|-------------------------|---------------------------|------------------|
| 1                   | 40          | 27                      | 21                        | 67.50%           |
| 2                   | 40          | 31                      |                           |                  |

Table 2: Numerical data for transformation experiments in the field

| Exp. No. | No. of embryos isolated | Agrobacterium treated embryos | Embryos on MS plates | Died | Selection tubes | Plantlets died | Plants transferred to pots | plants died in pots | Plants shifted to greenhouse |
|----------|--------------------------|-------------------------------|----------------------|------|-----------------|-----------------|---------------------------|----------------------|---------------------------|
| 1        | 403                      | 403                           | 403                  | 396  | 7               | 1               | 6                         | 1                    | 5                         |
| 2        | 345                      | 345                           | 345                  | 341  | 4               | 2               | 2                         | 1                    | 1                         |
| 3        | 314                      | 311                           | 311                  | 307  | 4               | 2               | 2                         | 1                    | 1                         |
| 4        | 310                      | 305                           | 305                  | 298  | 7               | 1               | 6                         | 3                    | 3                         |
| 5        | 350                      | 345                           | 345                  | 340  | 5               | 2               | 3                         | 0                    | 3                         |
| 6        | 409                      | 410                           | 410                  | 404  | 6               | 2               | 4                         | 2                    | 2                         |
| 7        | 320                      | 319                           | 319                  | 314  | 5               | 2               | 3                         | 1                    | 2                         |
| 8        | 410                      | 410                           | 410                  | 404  | 6               | 3               | 3                         | 1                    | 2                         |
| 9        | 311                      | 311                           | 311                  | 309  | 2               | 1               | 1                         | 0                    | 1                         |
| 10       | 336                      | 335                           | 335                  | 330  | 5               | 2               | 3                         | 1                    | 2                         |
| 11       | 400                      | 410                           | 410                  | 406  | 4               | 1               | 3                         | 2                    | 1                         |
| 12       | 315                      | 312                           | 312                  | 310  | 2               | 1               | 1                         | 1                    | 0                         |
| Total    | 4223                     | 4216                          | 4216                 | 4159 | 57              | 20              | 37                        | 14                   | 23                        |

Table 3: Transformation efficiency

| Agrobacterium treated embryos | Control plants | Plants shifted to green house | Transformation efficiency |
|-------------------------------|----------------|-------------------------------|---------------------------|
|                               | Control       | Experimental                  |                           |
|                               | plants         |                               |                           |
| 4216                          | 50             | 23                            | 21                        | 46%             |
|                               | 0.49%          |                               |                           |