DEVELOPMENT OF STEM RUST RESISTANT TRANSGENIC WHEAT PLANTS THROUGH THE UTILIZATION OF THE *PR2* GENE

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ABSTRACT

Wheat (*Triticum aestivum* L.) productivity is severely constrained by biotic stresses, among which stem rust remains a major threat to global food security. In this study, we developed wheat line overexpressing the pathogenesis-related gene 2 (*PR2*) gene to build innate antifungal defenses. The *PR* gene coding sequence was cloned into the pCB-*PR2*-P2A-GUS expression vector and introduced into embryogenic calli of the TD-1 cultivar via biolistic transformation. Regenerated plantlets were subjected to rigorous molecular analysis, including PCR and RT qPCR, confirming stable genomic integration and ~35-fold upregulation of *PR2* gene transcripts relative to non-transgenic controls. Histochemical β-glucuronidase (GUS) assays revealed spatial and temporal expression patterns across callus and developing seeds, indicating robust promoter-driven activity. Functional evaluation under natural pathogen exposure demonstrated marked reduced stem rust pustule formation in transgenic lines, validating the antifungal efficacy of β-1,3-glucanase in plants. *In silico* allergenicity analysis showed minimal similarity to known wheat allergens, supporting the biosafety of the transgene. These results established a comprehensive framework for *PR2* gene-mediated disease resistance, integrating molecular validation with phenotypic assessment. Future field trials will elucidate the durability of stem rust resistance and yield stability, providing strategic insights for wheat breeding programs.

Keywords: Stem resistance, *PR2* gene, Transgenic wheat, Wheat transformation

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INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops and serves as a major staple food for a large proportion of the global population. It contributes substantially to food security, particularly in developing countries where wheat-based products represent a primary dietary component (Ahmed *et al.,* 2022; Ammar *et al.,* 2023). Despite its importance, wheat production is increasingly threatened by multiple biotic and abiotic stresses that significantly limit crop productivity. In Pakistan, variations in climatic conditions, including temperature fluctuations and changing rainfall patterns, influence pest population dynamics and the prevalence of plant diseases, ultimately affecting wheat yield (Abbas *et al.,* 2022). Several fungal diseases, such as rusts, smuts, and blights, frequently occur in wheat-growing regions and can cause substantial yield losses when environmental conditions become favorable for pathogen development. Climate change has further intensified these challenges by altering disease distribution and increasing the risk of recurrent epidemics, thereby posing a serious threat to sustainable wheat production and global food security (Hossain *et al.,* 2024; Singh *et al.,* 2025).

Conventional breeding has historically played a significant role in improving wheat productivity and disease resistance; however, the complex hexaploid genome of wheat and the rapid evolution of pathogens often limit the effectiveness of traditional breeding strategies (Borrelli *et al.,* 2018). The integration of modern biotechnology with classical breeding approaches has therefore emerged as an important strategy for crop improvement. Genetic transformation technologies allow the introduction of specific genes that enhance plant defense responses and improve tolerance against biotic stresses (Chen *et al.,* 2022). Among the different transformation approaches, particle bombardment and Agrobacterium-mediated transformation have been widely used for the development of transgenic wheat plants (Zhuang *et al.,* 2024). Although transformation efficiency in wheat remains relatively low compared with other cereals such as rice and maize, continuous advances in plant tissue culture and molecular tools have significantly improved the feasibility of wheat genetic engineering (Zaidi *et al.,* 2019).

Pathogenesis-related (PR) genes play an important role in plant defense mechanisms against fungal pathogens. Among these, the *PR2* gene encodes β-1,3-glucanase, an enzyme capable of hydrolyzing β-1,3-glucans

that constitute a major structural component of fungal cell walls (Han *et al.*, 2024). These glucans act as microbe-associated molecular patterns (MAMPs) that are recognized by plant immune systems, triggering defense responses and the upregulation of *PR* genes during pathogen attack. Overexpression of *PR* genes has been reported to enhance resistance against various fungal pathogens by strengthening the plant's innate defense system (Lü *et al.*, 2022). Therefore, the introduction and expression of *PR* genes in susceptible crop varieties represent a promising strategy for improving plant protection and reducing yield losses caused by fungal infections (Dos Santos *et al.*, 2023). In this context, the present study aimed to introduce the *PR2* gene from a high-yielding wheat cultivar into a susceptible wheat cultivar and to regenerate transgenic plants through tissue culture techniques, followed by molecular confirmation of gene integration and evaluation of its potential antifungal activity *in vitro*.

MATERIALS AND METHODS

Plant Material: Seeds of 92 wheat (*Triticum aestivum* L.) cultivars (Table S1: List of wheat cultivars) were obtained from the Molecular and Medical Genetics Laboratory, Government College University Faisalabad (GCUF), Pakistan, and grown under contained conditions. The rust-resistant cultivar Chenab-70 (Numan *et al.*, 2021), carrying the *PR2* gene, was used as the donor genotype, whereas high-yielding but rust-susceptible cultivar TD-1 (Samon *et al.*, 2022) was considered as recipient genotype for transformation experiments. Among the tested genotypes, TD-1 exhibited superior embryogenic potential and regeneration efficiency; therefore, this cultivar was selected as recipient genotype for subsequent transformation experiments.

DNA Extraction: Genomic DNA was extracted from leaf tissue for molecular analysis according to Allen *et al.* (2006). Fourteen days old leaves were taken from plants and washed with distilled water to clean the surface and eliminate any dust particles. The cetyl trimethyl ammonium bromide (CTAB) method was employed for DNA extraction, and the resultant DNA was analyzed using a 1% agarose gel prior to quantification with the Nanodrop manufactured by ThermoFisher Scientific (Yu *et al.*, 2017).

***PR2* Gene Screening:** Genomic DNA from 92 locally available wheat cultivars was screened for the presence of the *PR2* gene using gene-specific primers through conventional PCR. Gene-specific primers were designed for the screening of the *PR2* gene using an online PrimerQuest tool (https://eu.idtdna.com/Primerquest/Home/), and all wheat varieties were screened for the *PR2* gene by performing conventional PCR (Madenova *et al.*, 2021).

RNA Isolation and cDNA Synthesis: Total RNA was extracted from the stem rust-resistant wheat cultivar Chenab-70, which served as the donor genotype carrying the *PR2* gene according to Chomczynski *et al.* (1987); Sambrook *et al.* (2001). First-strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) and used as a template for the amplification and isolation of the full-length *PR2* gene.

Total RNA was also extracted from the stem rust-susceptible recipient wheat cultivar, i.e., TD-1, to validate RNA extraction and cDNA synthesis procedures. This genotype was used as negative control to verify the specificity of *PR2* gene-specific PCR primers. First-strand cDNA was synthesized using RevertAid Reverse Transcriptase and subsequently used as a template for PCR analysis to confirm the absence of *PR2* gene amplification.

PCR Amplification from Chenab-70: The full-length *PR2* gene was amplified from the Chenab-70 donor cultivar again using forward and reverse primers with *Xba*I (Cat. # ER0681) and *Bam*HI (Cat. # ER0051) of Thermo Fisher Scientific™ restriction sites, respectively. The amplified full-length *PR2* gene was purified using Thermo Scientific GeneJet Gel Extraction Kit (Catalog # K0691) and stored at -20°C for further downstream applications (Numan *et al.*, 2021).

Table 1. The sequence of primers used for the amplification of the full-length *PR2* gene

Primer name	Primer sequence	No. of Bases	Restriction enzyme site
PR2-F1	5'-AATCTAGAATGGCTGGAAAGGATGTTGC-3'	28	*Xba*I
PR2-R1	5'-GCGGATCCGAACTGGATGTTGTAGGCCG-3'	28	*Bam*HI

Construction of Recombinant Expression Vector: The amplified *PR2* gene fragment and the pCB-P2A-GUS vector were digested with restriction enzymes. Afterwards, the full length *PR2* gene and linear pCB plasmid were ligated using the Thermo Fisher Scientific T4 DNA ligase kit (Catalog # EL0011) (Sambrook *et al.*, 2001; Wang *et al.*, 2023)

Transformation of Recombinant Plasmid into *Escherichia coli* (*E. coli*): The vector pCB-*PR2*-P2A-GUS was transformed into the freshly prepared competent cells of *E. coli* DH5α as described by Chen *et al.* (2001); Sambrook *et al.* (1989) with the following specified modifications: 30 minutes pre-incubation of competent cells at 4°C, use of 5 µL ligation mixture with 20 µL competent cells, 90 seconds heat shock at 42°C, 2–3 minutes

recovery on ice, and 1hour recovery in 200 µL of S.O.C. medium at 37°C before selection. After the 30 minutes incubation at 4°C, 5 µL of the ligation reaction was added to 20 µL of competent E. coli DH5α strain cells. The cells were exposed to 42°C for 90 seconds, followed by a brief immersion on ice (approximately 2–3 minutes). The transformed cells were then resuspended in 200 µL of S.O.C. medium and incubated at 37°C for 1 hour. Subsequently, the cells were cultured overnight at 37°C on LB agar plates containing 100 µg/mL ampicillin. A single colony was selected and inoculated into 5 milliliters of LB broth with 100 µg/mL ampicillin, and the culture was incubated at 37°C overnight.

Confirmation of Recombinant Plasmid: Plasmid DNA was extracted from positive bacterial colonies using Thermo Scientific GeneJET Plasmid Miniprep Kit (Cat. # K0502) and subjected to restriction digestion analysis using *Xba*I and *Bam*HI enzymes. The digestion products were analyzed on agarose gel to confirm the successful insertion of the *PR2* gene into the vector (Zhang *et al.*, 2018).

Callus Induction from Wheat Mature Embryos: For plant transformation experiments, mature embryos of wheat cultivar TD-1 were excised and cultured on Murashige and Skoog (MS) medium supplemented with 2,4-D for callus induction (Kumar *et al.*, 2017). The wheat cultivar TD-1 was selected due to its high tissue culture regeneration potential, which is critical for successful genetic transformation in wheat by Iqbal *et al.* (2016); Rashid *et al.* (2009). Embryogenic calli were maintained under controlled growth conditions for subsequent transformation.

Biolistic Transformation: Transformation was performed using a gene gun (PDS-1000/He system, Bio-Rad, USA). Gold particles were coated with plasmid DNA containing the *PR2* gene using spermidine and $CaCl_2$. The coated particles were loaded onto micro-carrier membranes and bombarded onto wheat calli under helium pressure of 1100–1350 psi at a target distance of 6 cm, as described earlier by Hamada *et al.* (2017).

Selection of Transformed Calli: Following transformation, calli were transferred to MS medium containing different concentrations of kanamycin (0–100 mg/L) to determine inhibitory concentrations. Transformed calli were selected on medium containing 50 mg/L kanamycin and incubated under controlled growth conditions by Tran *et al.* (2015).

Plants Regeneration

Callus-to-Shoot Formation: The regeneration medium was prepared according to Kumar *et al.* (2017). For shoot induction, MS medium supplemented with TDZ (1 mg/L), 2,4-D (0.1 mg/L), and kanamycin (50 mg/L) was dispensed (10 mL) into sterile Pyrex test tubes. Embryogenic calli (~50 mg) were aseptically transferred onto the regeneration medium, with one callus piece placed in each test tube. Cultures were maintained at 25 ± 2°C under controlled conditions. Sub culturing was performed weekly for three weeks following the protocol of Ishida *et al.* (2015).

Root Formation: Rooting medium was prepared and optimized using MS basal medium supplemented with NAA (0.5 mg/L) and kanamycin (50 mg/L). Regenerated shoots measuring approximately 3–5 cm in length were excised and transferred to the rhizogenesis medium for root induction. The cultures were maintained under the same growth conditions until well-developed roots were formed by follow the protocol of Tran *et al.* (2015).

Acclimatization of Regenerated Plants: Rooted plantlets were carefully removed from culture medium, washed with distilled water, and transferred into pots containing sterilized sandy loam soil. Plants were maintained under greenhouse conditions for hardening and further growth according to Kumar *et al.* (2017) with slight modifications.

Molecular Confirmation of Transgenic Plants

PCR Confirmation: Transgenic plants were further tested for their successful gene transformation. For this purpose, DNA from fresh wheat leaves was extracted, and PCR analysis was performed for the confirmation of gene transfer. The sets of primers are listed in Table 2. The PCR reaction consisted of an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 93°C for 30 seconds and annealing at 60°C for 1 minute, with a final extension at 72°C for 5 minutes and a hold at 4°C for infinity.

Table 2. The sequence of primers used for the confirmation of the *PR2* gene through PCR

Primer name	Primer sequence	Product size (bp)
KanR-F	5’-ACTCTCAACTCGATCGAGGCAT-3’	508
KanR-R	5’-TGATGCTCTTCGTCCAGATCATC-3’	
35S-*PR2*-F	5’-AATCCCACCCCTACTCCAAAA-3’	968
35S-*PR2*-R	5’-TCATGTACGACTGCGCGAA-3’	

Expression Analysis: Expression levels of the *PR2* gene in transgenic plants were quantified using RT qPCR. Relative expression levels were calculated using the ΔΔCt method, with appropriate reference genes used for normalization. Quantitative PCR was performed using the CFX96 Real-Time PCR system (Bio-Rad).

Table 3. The sequence of primers used for the confirmation of *PR2* gene through RT qPCR

Primer name	Primer sequence	No. of Bases
PR2-qF	5'- AACAATGTCCGGCCATACTAC-3'	21
PR2-qR	5'- CACCTTGATGGCGCTGA-3'	17

Quantitative PCR was performed with a total reaction volume of 10 µL. First-strand cDNA was synthesized from 1 µg of total RNA using RevertAid Reverse Transcriptase at 50–55°C for 30 minutes. The PCR reaction consisted of an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 93°C for 30 seconds and annealing at 60°C for 1 minute, with a final extension at 72°C for 5 seconds and a hold at 4°C for infinity. Relative gene expression levels were calculated using the ΔΔCt method.

Histochemical GUS Activity Assay: To investigate the expression of β-glucuronidase (GUS) encoding *UidA* gene in the putative transgenic wheat calli, the calli were incubated in X-Gluc buffer overnight at 37°C according to Barcelo *et al.* (1995). The X-Gluc buffer (10 mL) was prepared containing 0.5 mM potassium ferrocyanide, 100 mM sodium phosphate of pH 7.0, 1 mM X-Gluc, and 0.1% (v/v) Triton X-100.

Allergen Sequence Determination: The Structural Database of Allergenic Proteins (SDAP) (https://fermi.utmb.edu/SDAP/sdap who.html) was used to investigate the allergenic potential of β-1, 3-glucanase protein. All the alignments were made using the FASTA 3.45 version. All matching allergens were further searched for the source organism of the allergen in an online database following Mishra *et al.* (2012).

Antifungal Confirmation by Field Evaluation: Transgenic wheat plants expressing the *PR2* gene and non-transgenic control plants (TD-1) were grown under controlled greenhouse conditions to evaluate resistance against stem rust (*Puccinia graminis* f. sp. *tritici*). Plants were maintained under identical environmental conditions, and disease development was monitored under natural pathogen exposure. The field trials were conducted at Ayub Agricultural Research Institute, Faisalabad, Pakistan. Disease assessment was carried out through visual observation of symptom development, focusing on the presence and spatial distribution of uredinial pustules and overall disease progression, in accordance with standard wheat rust evaluation practices (Peterson *et al.,* 1948; Roelfs *et al.,* 1992).

RESULTS

Genomic DNA was extracted from 92 locally available wheat cultivars, and integrity was confirmed by 1% agarose gel electrophoresis (Fig. S1). DNA concentrations are summarized in Table S1. Conventional PCR using gene-specific primers revealed the presence of the *PR2* gene in only one genotype, Chenab-70 (Fig. 1, Lane 01). This genotype was selected as the donor for full-length *PR2* gene isolation. Subsequent PCR amplification showed no detectable *PR2* gene transcripts in the susceptible wheat cultivar (TD-1) used as negative controls, whereas the rust-resistant cultivar Chenab-70 served as the positive control and produced the expected amplification of 325 bp. This differential amplification confirmed the specificity of the designed primers and validated the efficiency of the cDNA synthesis procedure. These negative controls ensured that Chenab-70 was the appropriate source genotype for downstream cloning.

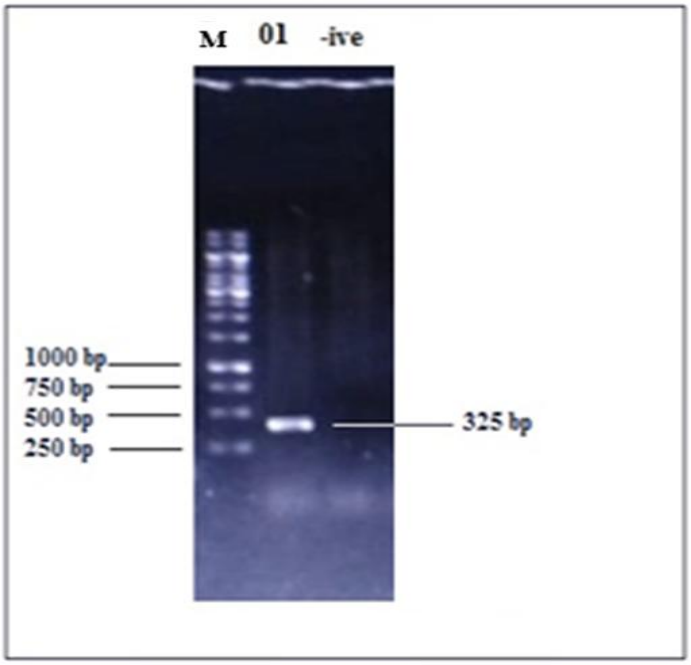

Figure 1. Screening of the *PR2* gene from local wheat varieties. Lane M=1 kb DNA ladder, Lane 01 = *PR2* gene from Chenab-70. Lane -ive = (TD-1).

Isolation of Full-Length *PR2* Gene: RNA was extracted from both donor (Chenab-70) and recipient (TD-1) following Chomczynski *et al.* (1987); Sambrook *et al.* (2001). Concentrations were determined using a NanoDrop spectrophotometer, and the results are presented in Table S2. PCR amplification from Chenab-70 cDNA yielded a single band corresponding to the full-length *PR2* gene (~1005 bp) (Fig. 2).

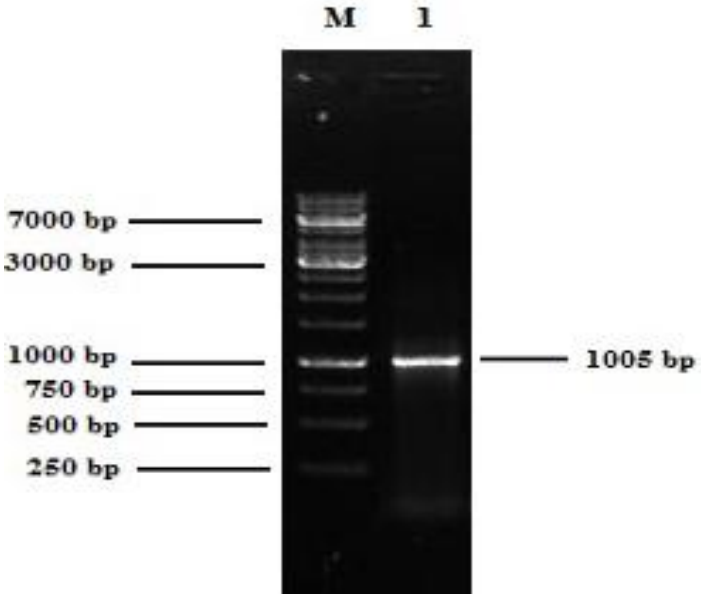

Figure 2. Full-length *PR2* gene isolation. M = 1 kb DNA ladder, and Lane 1 = *PR2* gene.

Construction of the *PR2* Gene Expression Vector: The *PR2* gene was cloned into the pCB-P2A-GUS expression vector, which includes the CaMV 35S promoter, GUS reporter gene, NOS terminator, and selectable marker genes for kanamycin and ampicillin resistance (Fig. 3). The circular map of the vector illustrates the positions of the *PR2* gene insert, *GUS* gene, regulatory elements, and restriction sites used for cloning.

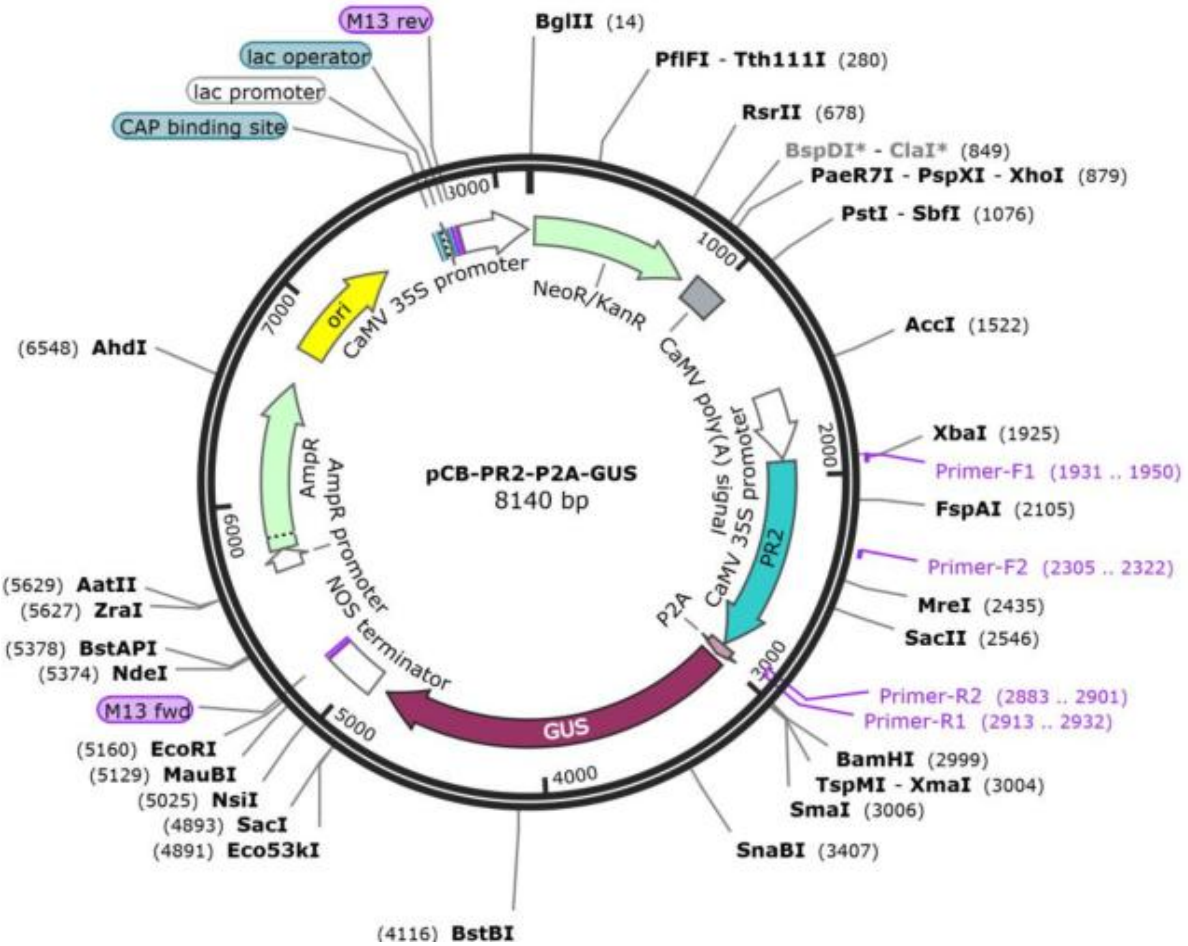

Figure 3. *PR2* gene cloning in pCB-P2A-GUS. Vector carrying the *PR2* gene under the CaMV 35S promoter with the GUS reporter and selectable marker genes (Kanamycin, Ampicillin, and Neomycin).

Confirmation of Vector by Restriction Digestion: Insertion of the *PR2* gene into the pCB-P2A-GUS vector was confirmed by restriction digestion. Digested plasmids with *Bam*H1 and *Xba*1 produced fragments of expected sizes (~7 kb linearized vector and ~1 kb *PR2* gene insert) (Fig. 4).

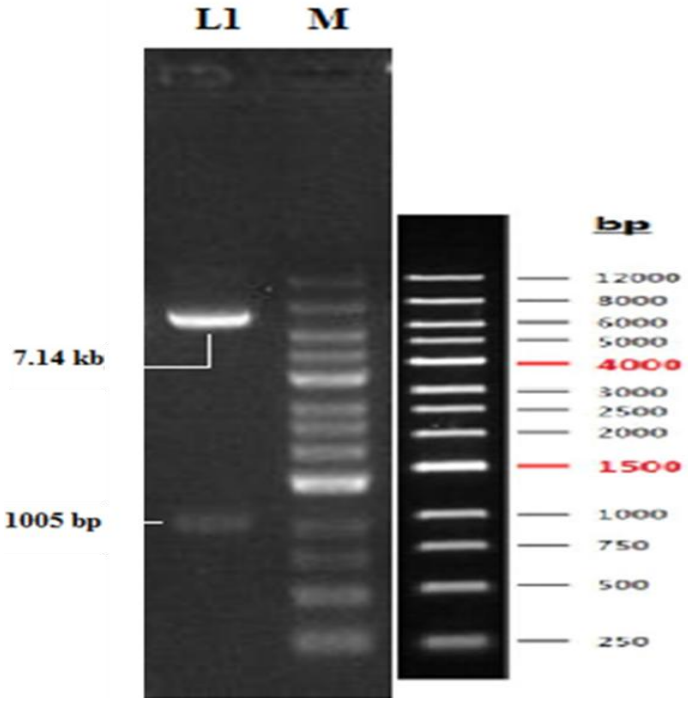

Figure 4. Restriction digestion of pCB-*PR2*-P2A-GUS. Lane M = 1 kb DNA ladder, and lane 01= clone plasmid DNA after digestion with *Bam*H1 and *Xba*1.

Callus Induction from Mature Wheat Embryos: Among six wheat varieties evaluated, TD-1 exhibited the highest callus induction (88%), while others showed poor embryogenic response.

Screening of Callus on Different Doses o*f Kanamycin:* Different concentrations of kanamycin were tested to determine the optimal selection pressure for transformed calli. Calli grown on medium without kanamycin (0 mg/L) showed normal growth and regeneration. Moderate inhibition of callus growth was observed at 30 mg/L kanamycin after 30 days of incubation. At 50 mg/L, approximately 50% inhibition of callus growth was observed. Concentrations above 70 mg/L resulted in strong inhibition, with calli drying and dying within 10–12 days (Fig. 5).

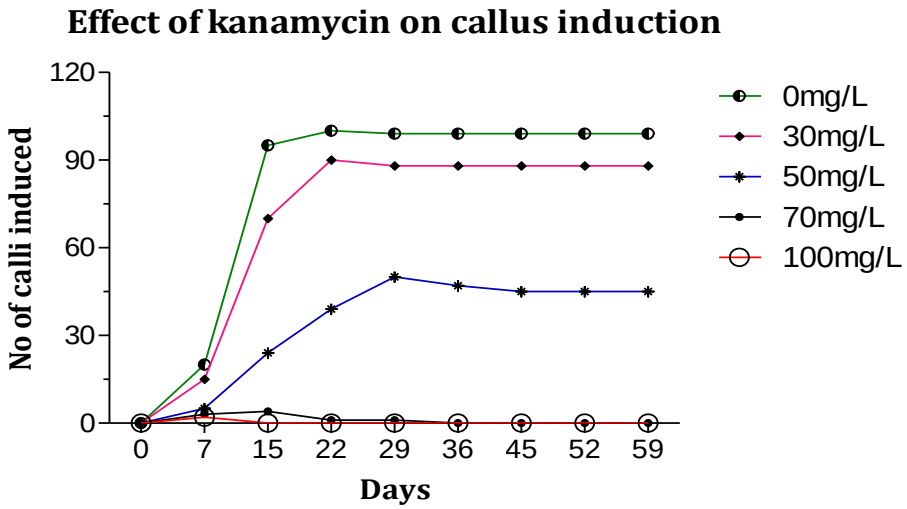

Figure 5. Effect of different doses of kanamycin on callus induction.

Biolistic Transformation of Wheat Calli*:* For transformation, embryogenic calli were pretreated on an osmotic medium containing half-strength MS supplemented with 256 g/L sucrose. This treatment resulted in partial dehydration of the calli and a color change to light brown (Fig. 6A). Following particle bombardment using the pCB-*PR2*-P2A-GUS plasmid, calli were transferred to recovery medium for 12 hours to allow tissue recovery (Fig. 6B). The treated calli remained viable and continued growth on the culture medium.

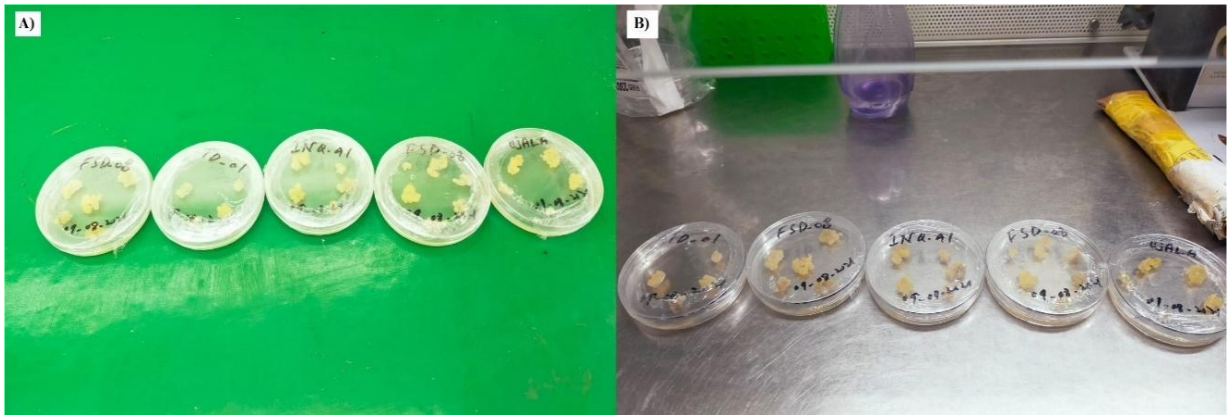

Figure 6. Wheat callus before and after pCB-*PR2*-P2A-GUS transformation (A & B, respectively).

Regeneration and Acclimatization of Putative Transgenic Plants: Transformed calli were transferred to regeneration medium, where they developed shoots and roots after successive subcultures. Shoots measuring approximately 3–5 cm were transferred to the rooting medium for further development. The number of bombarded calli were 75, whereas 19 calli out of 75 bombarded calli were transformed as confirmed through PCR. The transformation efficiency was 25.33%. Furthermore, the total number of 10 calli were regenerated from transformed calli with an efficiency of 52.63 %.

Table 4. Transformation and regeneration data of wheat callus

Callus Initiation	Date of Bombardment	Wheat cultivar	No. of Bombarded calli	No. of Transformed calli	No. of Regenerated calli
August 9, 2021	September 11, 2021	TD-1	75	19	10

Regenerated plantlets were removed from culture medium, washed to remove residual media, and transferred to soil-filled pots for acclimatization. Plants were initially covered with polythene bags and maintained under controlled conditions for 15–20 days to facilitate hardening (Fig. 7A). After acclimatization, the plants were transferred to larger pots where they grew to the tillering stage (Fig. 7B).

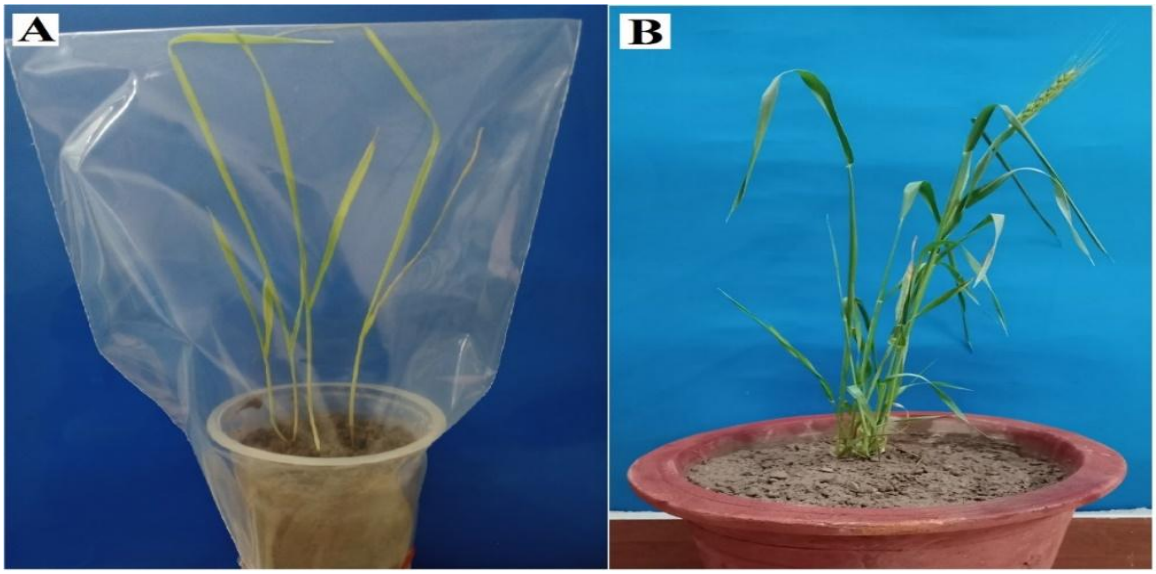

Figure 7. Regeneration and acclimatization of putative transgenic wheat plants. A) Hardening of regenerated plantlets in soil-filled pots; B) Growth of acclimatized plants to the tillering stage under greenhouse conditions.

Molecular Confirmation of Transgenic Plants: Transgenic plants were further tested for gene transformation; conventional PCR was performed on genomic DNA. Primer set KanR-F and KanR-R amplified the 508 bp long fragment, and primer set 35S-*PR2*-F and 35S-*PR2*-R amplified the 968 bp long DNA fragment (Figure 8).

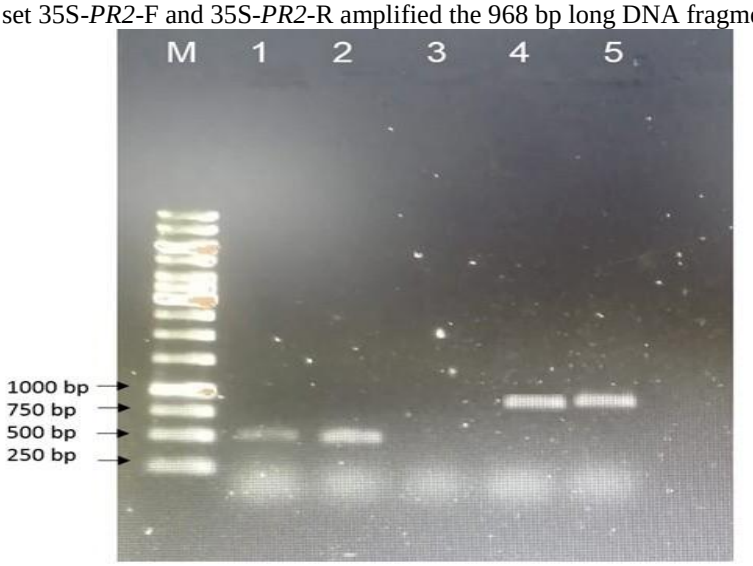

Figure 8. Confirmation of transgenic wheat plants through conventional PCR. Lane M=1kb Ladder, Lanes 1 and 2 (Amplification of kanamycin resistant gene, in duplication for authenticity) in TD-1; Lanes 4 and 5 (Amplification of 35S promoter , in duplication for authenticity), in TD-1, which confirmed the transformation of the gene of interest in the putatively developed plants. Lane 3 (non-transgenic, negative control), no amplification.

Expression Analysis of *PR2* Gene: The results of RT qPCR revealed the relative expression of the PR2 gene in putative transgenic and non-transgenic wheat Plant. Putative transgenic wheat plants exhibited approximately 35-fold higher PR2 gene expression compared with non-transgenic controls (Figure 9).

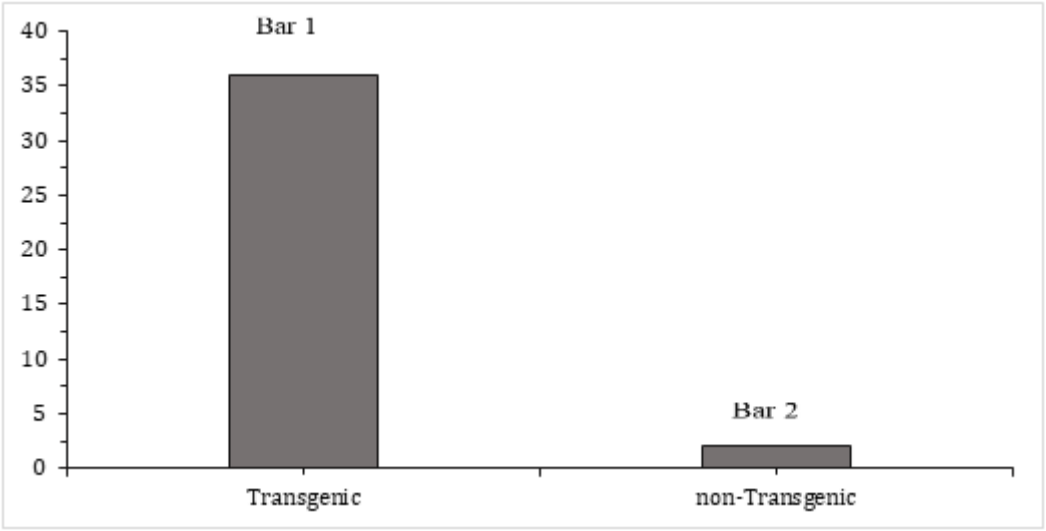

Figure 9. Relative *PR2* gene expression through RT qPCR. Bar 1 is showing the expression of the *PR2* gene in putative transgenic plants, and Bar 2 is showing relatively lower or no gene expression in non-transgenic plants of the same wheat cultivar, i.e., TD-1.

GUS Activity Assay: Histochemical β-glucuronidase (GUS) staining was performed to assess the expression of the pCB-*PR2*-GUS construct in transgenic wheat tissues. Blue coloration indicating GUS activity was detected in both transgenic calli at 15, 18, 21, 25, and 28 days post-transformation. GUS expression in 15 and 18 days old tissues was relatively weak and localized to specific regions of the callus and endosperm. However, tissues harvested at 21, 25, and 28 days post transformation showed progressively stronger staining, indicating increased transgene expression at later developmental stages. The highest GUS activity was observed at 28 days post transformation.

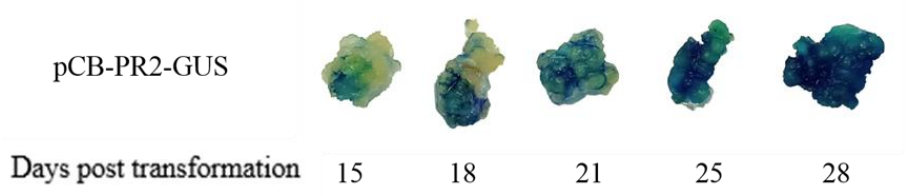

Figure 10: Histochemical staining for GUS activity in putative transgenic wheat calli. Transverse section of transgenic wheat calli harvested at 15, 18, 21, 25, and 28 days post-transformation.

Allergenicity Assessment: Allergenicity of the β-1,3-glucanase (*PR2*) gene was evaluated *in silico* in accordance with the FAO/WHO Codex Alimentarius guidelines for genetically modified crops (Codex Alimentarius Commission, 2003) and as detailed in Mishra *et al.* (2012). The amino acid sequence was subjected to similarity searches against established allergen databases, including FASTA-based full-length alignment, 80-amino-acid sliding window analysis, and exact match screening for contiguous amino acid stretches.

The full-length FASTA alignment identified three potential allergen matches, namely Hev b 2 from *Hevea brasiliensis* and Ole e 9 from *Olea europaea*, indicating moderate sequence homology. The 80-amino-acid sliding window analysis yielded a total of 255 hits, including 21 matches corresponding to Hev b 2, with identity values ranging from 48.75–61.25%, suggesting localized regions of similarity within conserved domains of β-1, 3-glucanase proteins.

Furthermore, exact match analysis for six contiguous amino acids resulted in 30 hits, including allergens such as Hev b 2 (*Hevea brasiliensis*), Ole e 9 (*Olea europaea*), Chi t 6 (*Chironomus thummi thummi*), Peni c 13 (*Penicillium citrinum*), and Aed a 8 (*Aedes aegypti*). These matches are consistent with conserved short peptide motifs commonly observed among functionally related proteins and do not necessarily indicate allergenic potential.

Field Evaluation of Rust Resistance: Transgenic plants expressing the *PR2* gene exhibited reduced disease symptoms under natural pathogen exposure compared to non-transgenic controls, confirming functional resistance conferred by the transgene.

Figure 11. Field evaluation of stem rust resistance in wheat plants at Ayub Agricultural Research Institute, Faisalabad, Pakistan. **(A) Non-transgenic control plants showing severe stem rust infection. (B) *PR2* transgenic plants showing reduced disease symptoms.**

Under natural field conditions, non-transgenic plants exhibited clear rust symptoms with numerous orange pustules on the stems, whereas *PR2* gene transgenic plants showed markedly fewer symptoms. The reduced stem rust severity in transgenic lines indicated that *PR2* gene expression introduced resistance under natural pathogen exposure.

DISCUSSION

Wheat (*Triticum aestivum* L.**)** productivity is severely affected by stem rust disease caused by *Puccinia graminis* f. sp. *tritici*, posing a major threat to sustainable wheat production. In the present study, the stem rust-resistant wheat cultivar Chenab-70 carrying the *PR2* gene (Numan *et al.*, 2021) was selected as the donor genotype, whereas the high-yielding but susceptible cultivar TD-1 (Samon *et al.*, 2022) was used as the recipient

genotype due to its superior regeneration potential. The objective of this study was to develop transgenic wheat plants through biolistic-mediated transformation of the *PR2* gene and to evaluate its expression and potential role in enhancing stem rust resistance.

Screening of locally available wheat cultivars revealed the presence of the *PR2* gene in only one genotype, Chenab-70. This observation suggested substantial genetic variation among wheat cultivars with respect to defence-related genes. Similar variability in *PR*2 gene distribution and expression among wheat germplasm has been reported in earlier studies, indicating that genetic diversity influences the level of resistance against fungal diseases (Singh *et al.,* 2023). The identification of Chenab-70 as the source genotype for *PR2* gene isolation, therefore, highlights the importance of exploiting natural genetic resources for crop improvement. Amplification of the full-length *PR2* gene (~1005 bp) and confirmation through sequencing verified that the isolated gene corresponds to a functional β-1, 3-glucanase gene. Comparable gene lengths and sequences have been reported for *PR2* gene in wheat and other cereals, further supporting the identity of the isolated gene (Mohammadizadeh-Heydari *et al.,* 2024; Raji *et al.,* 2022).

Construction of the *PR2* gene expression cassette using the pCB-*PR2*-P2A-GUS vector was confirmed through restriction digestion. The use of the CaMV 35S promoter ensured constitutive expression of the transgene. Constitutive promoters have been widely used in plant genetic engineering to achieve stable and strong expression of defence-related genes. Expression of the *PR2* gene under strong promoters significantly enhances resistance against fungal pathogens in several crops, including tobacco, rice, and wheat (Pathania *et al.,* 2022). The vector system used in the present study, therefore, provided an effective platform for evaluating the functional role of the *PR2* gene in wheat.

Efficient plant regeneration is a critical factor in cereal transformation systems. Among the wheat cultivars evaluated in this study, TD-1 exhibited the highest callus induction, transformation efficiency (25.33 %) and regeneration efficiency (52.63 %). Previous studies have reported transformation efficiency of 25 % (Hayta *et al.,* 2019) and regeneration efficiencies ranging from 68–90% under optimized wheat tissue culture conditions (Rashid *et al.,* 2009; Iqbal *et al.,* 2016). The variation observed among genotypes is consistent with earlier reports that wheat transformation efficiency is highly genotype-dependent (Park *et al.,* 2022; Ye *et al.,* 2023). Certain cultivars possessed higher embryogenic potential and responded better to tissue culture conditions, which significantly influenced transformation success. The relatively higher regeneration efficiency of TD-1 observed compared with other cultivar possessed favourable physiological characteristics for *in vitro* regeneration and genetic transformation.

Molecular confirmation of transformed plants through PCR amplification of the kanamycin resistance gene and *PR2* gene fragment verified the successful integration of the transgene into the wheat genome. Furthermore, real time quantitative PCR analysis revealed approximately 35-fold higher expression of the *PR2* gene in transgenic plants compared with non-transgenic controls. This elevated expression level indicates effective transcriptional activity driven by the CaMV 35S promoter. Comparable increases in *PR2* gene expression have been reported in previous studies, in which transgenic plants expressing β-1,3-glucanase genes showed improved resistance against fungal pathogens (Balasubramanian *et al.,* 2012; Sharma *et al.,* 2021). The increased transcription observed in this study, therefore, confirmed the functional activity of the introduced gene.

Progressive introduction of GUS staining during later stages indicates stable transgene expression across post transformation stage. Reporter genes such as β-glucuronidase have been extensively used in plant biotechnology to monitor promoter activity and transgene expression patterns. GUS expression patterns in transgenic cereals, where reporter gene activity increased during later stages of plant development (Jefferson *et al.,* 1987). The strong staining observed in the present study confirmed stable integration and expression of the *PR2*-GUS construct in wheat tissues.

Field observations provided preliminary evidence that *PR2* gene expressing transgenic plants exhibited reduced rust symptoms compared with non-transgenic controls. Non-transgenic plants showed typical orange uredinial pustules characteristic of infection by the wheat stem rust pathogen *Puccinia graminis* f. sp. *tritici*, whereas *PR2* gene expressing plants displayed markedly fewer symptoms under the same environmental conditions. B-1, 3-glucanases encoded by *PR2* gene were known to degrade β-glucans present in fungal cell walls, thereby inhibiting fungal growth and strengthening plant defence responses. Previous studies have reported that overexpression of *PR* genes can significantly enhance resistance against fungal pathogens in several crop species (Grover *et al.,* 2003; Lü *et al.,* 2022). The reduced rust severity observed in the present work, therefore, supports the proposed antifungal role of *PR2* gene in wheat defence.

In addition to evaluating disease resistance, allergenicity assessment was performed to determine the biosafety of the expressed *PR2* gene product. Bioinformatic analyses indicated limited sequence similarity between the β-1,3-glucanase protein and known allergens from non-wheat sources, particularly Hev b 2 and Ole e 9, which belong to the same conserved protein family. Importantly, no wheat-derived allergenic sequences exhibited significant identity (>35%) with the *PR2* protein, suggesting minimal risk of cross-reactivity with endogenous wheat allergens. Although sequence homology is often considered indicative of potential allergenic cross-reactivity, it does not necessarily translate into clinically relevant allergenicity, as proteins sharing 45–55%

identity have been reported to lack immunological reactivity under experimental conditions (Beyer *et al.,* 2001). Therefore, the observed similarities are more likely attributable to conserved structural domains of pathogenesis-related proteins rather than true allergenic potential. These findings are consistent with previous reports indicating that *PR* gene generally exhibit low allergenicity despite their central role in plant defence mechanisms (Goodman, 2008; Mishra *et al.,* 2012). Consequently, while the *in-silico* assessment suggests a low allergenic risk, further experimental validation through immunological assays is required to confirm these predictions. Collectively, the integration of *PR2* gene mediated resistance with a favourable safety profile highlights its potential for the development of disease-resistant wheat cultivars. Given the substantial yield losses associated with rust diseases globally, the deployment of *PR2* gene based genetic strategies represents a promising and sustainable approach for improving wheat productivity.

Conclusion: In this study, we developed transgenic wheat line expressing the *PR2* gene, which conferred resistance against stem rust. Functional antifungal activity was confirmed through field evaluation, demonstrating reduced disease symptoms compared with non-transgenic controls. These findings provide a promising foundation for further field studies in controlled conditions and detailed analysis to validate the effectiveness and stability of *PR2* gene mediated resistance under diverse environmental conditions.

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