GENETIC ENGINEERING OF WHEAT (*Triticum aestivum* L.) FOR ENHANCED FUNGAL DISEASE RESISTANCE

The large and complex genome, genotype-dependent tissue culture response and transgene silencing are the major hurdles for efficient genetic transformation of wheat. It was the last among the cereals to be genetically transformed. The transformation efficiency of this crop is less than other cereals transformed so far. In the present study, attempts were made to engineer the hexaploid wheat genome using both the Indian winter wheat cultivars (PBW343 and HD2687) as well as a Mexican spring wheat cultivar (Bobwhite), and the two genes coding for the antifungal molecules, viz glucose oxidase (GOX) from *Aspergillus niger* and antimicrobial protein Ace-AMP1 from *Allium cepa*. The oxidation reaction of glucose by GOX generates \( \text{H}_2\text{O}_2 \), as a by-product, which has a versatile role in the manifestation of plant defense response. It has been implicated in the reinforcement of structural barriers, induction of plant defense genes, mediation of hypersensitive response (HR) and programmed cell death (PCD) and in establishing systemic acquired resistance (SAR). Potato, tobacco, canola, cotton, cabbage and rice have been successfully transformed with the gene for GOX.

*Ace*-AMP1 belongs to the group of lipid transfer proteins with sequence homology and structural analogies to plant non-specific lipid transfer proteins (ns-LTPs). In contrast to other ns-LTPs like those isolated from radish and maize, *Ace*-AMP1 is unable to transfer...
phospholipids from liposomes to mitochondria due to the presence of aromatic residues in the domain corresponding to a lipid binding pocket found in true lipid transfer proteins. The protein is active against numerous fungi at concentrations ≤ 10 μg / ml but the underlying mechanism of action is still unclear. However, Ace-AMP1 has been successfully engineered into scented geranium, rose and very recently into indica rice where it has been shown to confer resistance against an array of phytopathogens.

To start with, genetic transformation of wheat various parameters of particle bombardment such as the amount of DNA, amount of gold particles, projectile distance etc. for the Indian wheat cultivars were optimised. Effect of various amounts of DNA was tested from 3 to 9 μg range. Amount of gold particles per shot was varied between 50 μg to 2000 μg. Optimum projectile distance was varied between 6 to 12 cm. In the transient transformation experiments, it was found that increasing amount of DNA did not have any drastic effect and 1250 μg of gold particle coated with 3 μg of DNA was sufficient to get satisfactory transient gene expression with a projectile distance of 8 cm.

In the transient genetic transformation experiments, Cl, Bperu (transcription factors needed for anthocyanin biosynthesis) and gus genes were used. These experiments were performed for both Indian, Mexican and Swiss wheat cultivars (‘Frisal’, ‘Golin’ and ‘Greina’). Throughout the course of genetic transformation work ~3,000 immature wheat embryos were isolated for this purpose and ~2,000 of these produced somatic embryos suitable for ballistic gene delivery. A large number of red spots distributed all over the
scutellar surface could be observed when the bombardment parameters were optimised for the Particle Inflow Gun (PIG).

For the stable transformation experiments, in order to ensure higher probability of getting the transgene of interest (either \textit{GOX} or \textit{Ace-AMP1}) integrated in the genome the respective constructs, for the transgene of interest, were taken in higher molar ratio (from 2:1 to 5:1) as compared to the selectable marker. As controls, the non-bombarded calli and the calli that were bombarded with uncoated gold particles were incubated on selection and/or regeneration medium, for each set of experiment.

Co-bombardment experiments was performed using \textit{pPAL::Ace-AMP1} and \textit{pAct::BAR} for PBW343 and \textit{pUbI::Ace-AMP1} and \textit{pAct::BAR} for both HD2687 and Bobwhite. In total, 1501 PBW343, 1503 HD2687 and 595 Bobwhite immature embryos were isolated to be used as explants, out of which, 1137, 1215 and 595 generated somatic embryogenic calli, respectively, suitable for bombardment. After selection and regeneration, 331 phosphinothricin resistant planlets (32 for PBW343, 96 for HD2687 and 203 for Bobwhite) could be obtained and finally 113 \textit{T}0 plants could grow up to maturity.

\textit{Bar} expression analysis was carried out on the primary transformants using chlorophenol red assay and tolerance of the transformants to the herbicide bialaphos was tested by leaf paint assay in the \textit{T}1 and \textit{T}2 generations. The primary transformants which showed positive results for higher concentrations of phosphinothricin (10 mg/l) in the chlorophenol red assay were selected for molecular analysis. In the leaf paint assays, the
transgenic plants remained unaffected but the null segregants showed yellowing and subsequently the leaf tissue showed necrosis. Many of the primary transformants turned out to be “escapes” and the absence of the selectable marker bar was confirmed by PCR.

Inheritance of the transgene of interest was monitored using PCR and transgenic lines were identified with the amplification of transgene products of 363 bp for Ace-AMP1 and 444 bp for bar. In the T1 generation, 10 seeds were germinated for various wheat transformants and were pre-screened for the presence of transgenes. Three transformants which showed higher levels of expression of bar and where the gene for Ace-AMP1 could be detected were selected for further analysis. Presence of both the transgenes was further tested in the T2 generation taking 10 offspring for each line. Although the 'Bobwhite' transformants could be identified, no transformed Indian wheat line could be detected. The co-transformation efficiency for 'Bobwhite' was 60%, for bar and Ace-AMP1.

PCR results were confirmed by Southern blot analysis in the T1 and T2 generations using the coding sequences of bar and Ace-AMP1 as probes and the analysis uncovered the integration patterns of the transgenes in the wheat lines BW235.15, BW237.5 and BW240.6 tested. The DNA from the untransformed plant and null segregant was used as negative control in the T1 and T2 generations, respectively. Hybridisation signals of different complexities indicated the occurrence of different transformation events. Hybridisation for the gene for bar showed three different Southern profiles with number of integrations varying from 2 to 4 in various individuals in the T1 generation. Hybridisations for the individuals in the T2 generation identified 6 different Southern
profiles with at least 4 integrations in majority of the cases. Hybridisation patterns of different complexities were also obtained when the respective blots were hybridised with the probe for Ace-AMP1. T1 generation individuals for the three lines BW235 15, BW237 5 and BW240 6 showed at least 5 different Southern profiles indicating different transformation events. In the T2 generation, Southern blot analysis of individuals from respective transgenic lines demonstrated 3 different hybridisation patterns but largely the banding profile was consistent to what was observed in the previous generation. However, in some cases, a few truncations of the transgene cassette were also observed.

RT-PCR analysis was carried out using primers specific for the mRNA sequence of Ace-AMP1 to monitor the expression of the gene at the mRNA level. Majority of the individuals from the lines BW235 15, BW237 5 and BW240 6 showed expression of the transgene product of 363 bp, in the T1 generation. The RT-PCR results were confirmed by Northern blot analysis using the coding sequence of Ace-AMP1 as the probe. RT-PCR and Northern blot analysis for the transgene was also performed in the T2 generation to investigate the stability of expression. Although, the number of plants exhibiting transgene silencing was less, progressive gene silencing phenomenon was observed for the transgene of interest in the transgenic line BW237 5 across the transgenic generations.

Immunoblot analysis detected stable expression of Ace-AMP1 protein in the transgenic lines BW235.15, BW237 5 and BW240.6 in both the generations with some variation in the levels of expression. The levels of Ace-AMP1 protein produced was also estimated quantitatively by means of indirect ELISA in the T2 generation. The amount of Ace-
AMP1 protein produced in various transgenic plants varied approximately from 2 to 4 µg / mg of total protein

The T2 generation plants were subjected to detached leaf assay using the spores of *B. graminis* f. sp *graminis* causing powdery mildew in wheat. A significant reduction was observed in the number of powdery mildew fungal colonies in the plants expressing *Ace*-AMP1, with respect to the non-transgenic plant, and up to 50% increase in resistance could be achieved. One way ANOVA indicated that there was a variation between the untransformed control and the transgenic lines with respect to the level of resistance achieved.

Furthermore, the spikes of transgenic wheat and null segregants were inoculated with *Neovossia indica*, causing Karnal bunt in wheat, to investigate whether *Ace*-AMP1 induced expression of defense-related genes. Total cellular RNA was isolated from the Karnal bunt inoculated ears of the line 235.15 and the null segregant, collected ½, 24, 48 and 72 hours after inoculation (hai). Northern blot analysis was performed for the genes for phenylalanine ammonia lyase (*PAL*), glucanase (*PR2*) and chitinase (*PR3*). Interestingly, increased levels of transcripts of *PAL*, *PR2* and *PR3* were observed. The fold difference in the expression of *PAL* transcript varied from 1.02 to 1.75 with highest level of induction reaching 24 hai. The transcripts for *PR2* (acidic isoform of β-1, 3-glucanase) and *PR3* (Class VII chitinase) could be detected as early as ½ hai in the transgenic line while for the null segregant induction of these genes could be observed only 24 hai. The highest level of induction of these genes was observed 24 hai (1.65-fold
for PR2 and 2.61-fold for PR3) in the transgenic line as compared to the null segregant. More interestingly, there was a progressive increase in the levels of salicylic acid (SA) in the wheat tissue until 72 hai in both transgenic wheat and the null segregant but levels of SA produced in the transgenic line was higher when compared to the null segregant. All these observations are indicative of manifestation of systemic acquired resistance (SAR) in wheat.

Agronomic parameters such as plant height, number of tillers per plant, days to 50% flowering, flag leaf length, length of panicles and percentage germination of the transgenic lines in the T2 generation were compared with those of the wild type 'Bobwhite' MANOVA helped conclude that there was a variation of the agronomic characteristics between the transgenics and the untransformed plant which could possibly be an effect of the activity of the transgene in the host system.

Further, in the effort to introduce the gene for GOX into the wheat system, in total, 3116 PBW343, 4911 HD2687 and 502 Bobwhite immature embryos were isolated to be used as explants, of which 2334, 3175 and 400 generated somatic embryogenic calli, respectively, suitable for bombardment. In the stable transformation experiments, for co-bombardment of embryos, the constructs pPAL::GOX and pAct::BAR were used for PBW343, HD2687 and Bobwhite and pUbI::GOX and pAct::BAR were used for PBW343 and HD2687. After selection and regeneration, 921 phosphinothricin resistant planlets (352 for PBW343, 531 for HD2687 and 38 for Bobwhite) were obtained and finally 165 T0 plants could grow up to maturity. Chlorophenol red assay and molecular
analysis involving polymerase chain reaction and Southern blot analysis were undertaken for the putative transformants in the T₁ generation individuals (660) have been tested from 66 different putative transformants. Although the gene for bar was present in most of the transformants, for presence of GOX could not be confirmed in any one of the transformants tested. This thing may be explained by the phytotoxic effects of GOX, in vivo. Phytotoxic effect of GOX was evaluated by external application of the enzyme at various concentrations ($10^{-10}$, $10^{-8}$, $10^{-6}$, $10^{-4}$, $10^{-2}$, 0.025, 0.05, 0.1, 0.5, 1.0 and 2.0 unit/µl) on the growing calli of rice and wheat. Stunted growth of the wheat calli was observed with progressive browning over the time at higher concentrations of GOX, respectively. However, at the same concentrations of GOX, the growth and development of the rice calli were not much affected except at very high concentrations of 1 to 2 unit/µl. The surviving wheat calli were further transferred onto regeneration medium and only the GOX untreated control calli were found to produce shoots.