Enhanced Drought Tolerance and Yield in Cry1ac and APX Co-Transformed Transgenic Rice (Oryza Sativa L.)

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Abstract

In the present study, transgenic cry1Ac-APX plants were developed in popular rice variety, BPT 5204, employing Agrobacterium LBA4404 harbouring pcam-cry1Ac-APX vector. The transgenic plants generated were analysed based on herbicide (Basta) tolerance and molecular analyses. Seeds of T1 generation when germinated on MS medium containing 4 mg/l phosphinothricin (ppt) revealed 3 tolerant: 1 susceptible plants suggesting that the transgenes showed monogenic segregation. Homozygous lines were identified by 100% germination of T3 seed on PPT containing media. Abiotic stress assays of analysis cry1Ac-APX transgenic lines showed enhanced chlorophyll, proline and reducing sugars compared to untransformed control plants. The antioxidant enzymes SOD and CAT showed significantly higher levels in transgenic lines than UC. RT-PCR analyses revealed increased expression levels of drought related genes OsDREB, OsMYB and bZIP under both stressed and without stress conditions. Further, the pcam-cry1Ac-APX lines exhibited enhanced biomass and yield than UC.

Introduction

Rice is among the major food crops consumed and is a staple diet for about two-thirds of the population across the world (Aebi 1984). In rice cultivation, India stood at second place with cultivated area of 43.39 million hectares and 104.3 million tons production. To meet its growing demand, India needs to increase its annual production to 333 million tons by 2050 against the present need of 252 million tons (Agricultural Statistics at a Glance 2016). Growth of Rice, its development and productivity are found to be intensely affected by different kinds of biotic and abiotic stress factors. Adverse environmental conditions like salinity, drought, and cold stresses cause loss of yield all over the world. Adaptation of the Plant to unfavourable environmental factors rely upon stress perception, signal transduction and stress-related genes expression. Reactive oxygen species (ROS) are generated as in the process of plant cellular metabolism and they aid as signalling molecules. Various abiotic stresses trigger cellular responses resulting in excessive production of ROS like hydrogen peroxide, hydroxyperoxyl, superoxide and oxygen radicals in the plants. Cell enters a state of “oxidative stress” when ROS levels outgrows the defence mechanism. Increased generation of ROS during environmental stresses induce enzyme inhibition, protein oxidation, nucleic acids damage, programmed cell death and lipid peroxidation pathways causing cell death and impacting the productivity and growth of plants (Sharma and Dubey 2005). Plants protect themselves from adverse stress conditions by activating different defence mechanisms like ascorbate, glutathione, tocopherols and ROS-detoxifying enzymes like catalases, peroxidases and superoxide dismutases (Inze and Van Montagu 1995).

Overexpression of drought tolerance related genes is one effective approach to develop drought resistant transgenics. Ascorbate peroxidase (APX; EC 1.11.1.11) is one of the important defense components and belongs to heme peroxidase in plants superfamily. The expression of APX gene is upregulated by various stress causing factors, implying that APX has a key role in the stress resistance of plants (Maruta et al. 2016). It has greater affinity towards peroxides (mainly \( \text{H}_2\text{O}_2 \)) and break down \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) and \( \text{O}_2 \) in the chloroplast, peroxisomes, and mitochondria by utilizing ascorbate as a specific electron donor (Sofo et al.
During biotic and abiotic stresses, APX plays key role in the intracellular ROS elimination and guards the plants from oxidative type of damage (Hong et al. 2018).

Insects and pests are few of the crucial problems to rice production in India. Majority of the insects belongs to hemiptera (sap-sucking) and also lepidoptera (usually stem borers and leaf folders) and it is very hard to control the insects in field conditions. The major pests, yellow stem borer such as Scirpophaga incertulius Walker, striped stem borer like Chilo suppressalis Walker and leaf folder such as Cnaphalocrosis medinalis Guenee, of lepidoptera cause 10–30% significant yield loss of rice, yearly (Li et al. 2018). Brown planthopper (BPH) mainly feeds on stems and take in phloem sap and thereby serves as a vector virus transmission causing reduction in the productivity of rice (Lu et al. 2016). Repeated use of chemical pesticides leads to lack of specificity effecting beneficial organisms, development of resistance upon prolonged application, besides, adverse impact on environment and human health. Insecticidal crystal protein genes referred as cry genes of Bacillus thuringiensis (Bt) are proved efficient towards pests and utilized in the field of plant genetic engineering. Genetic engineering of insect resistance genes from Bt has provided a safe, specific and effective approach to control insect pests (Snell et al. 2012). Bt toxins swallowed by insect get stimulated by the midgut proteases in order to develop crystals that disrupts the midgut wall, leading to insect death. The insecticidal activity of the bacterium is dependent on the capability to develop large quantity of delta-endotoxins (Cry proteins) (Manikandan et al. 2016). Since 1993, many transgenic rice plants were developed by transferring Bt genes including cry1Ab, cry1Ac and cry1B, conferred high resistance against lepidopteran pests (Cohen et al. 2008). Plants expressing cry1C gene proved resistant to pests that are actually resistant to Cry1A proteins and cry1A-cry1C genes combination resulted in killing more pests (Cao et al. 2012).

Materials And Methods

Bacterial strains and plasmids

The Bt and bacterial strains were taken from “The Microbial Type Culture Collection and Gene Bank” (MTCC), Institute of Microbial Technology, Chandigarh, India. Vectors, plasmids used in this study were picked up from Fermentas INC.

Isolation of cry1Ac gene from Bacillus thuringiensis:

The Bacillus thuringiensis culture was inoculated in LB broth medium and incubated overnight at 37°C on a shaker. Genomic DNA has been separated from the culture and used as PCR template according to reported method (Carozzi et al. 1991). The genomic DNA isolated from Bacillus thuringiensis used as template for PCR to amplify the full length cry1Ac gene using 5′–GGCCATATGGATAACAATCCGAACAT–3′ and 5′–CCCGGGCTATTCCTCCATAAGGAGTA–3′ as forward and reverse primers. The underlined regions in the primers indicate Ncol and SmaI restriction sites. The PCR product is electrophoresed in 0.8% agarose gel in 1X TAE buffer. The eluted PCR product of ~3537 bp is digested with Ncol and SmaI restriction enzymes and cloned between CaMV 35S promoter and poly(A) terminator of pRT100. The recombinant pRT100 vector harbouring cry1Ac was named as pRT100-cry1Ac. The recombinant clones of pRT100-
cry1Ac were further confirmed by Ncol and Smal restriction digestion. The cry1AC cassette (CaMV35S-cry1Ac-poly(A)) in pRT100-cry1Ac was excised using HindIII restriction enzyme and used for further ligating to APX cassette in the further steps.

Isolation of L-ascorbate peroxidase gene from rice plant

On the other hand, the fresh leaves of rice plant were selected and their total RNAs have been separated by making use of TRizol reagent. The synthesis of the first strand of the cDNAs was done by utilizing Prime Script™ RT reagent kit with g DNA eraser (Takara, Japan) as per the instructions by the manufacturer. cDNA has been utilized as a template and PCR was done using 5′–GGCCCATATGCTGATCTTTATCAGCTT–3′ and 5′–CCCGGGCTACTTGGTTTTCTTAGAAG –3′ as forward and reverse primers. The amplified ~516 bp fragment of L-ascorbate peroxidase gene was eluted from the agarose gel by freeze-thaw gel elution method using low-melting agarose (Sambrook and Russell 2001). The eluted ~516 bp of L-ascorbate peroxidase gene fragment was cloned between CaMV 35S and poly(A) of pRT100 vector at Ncol and Smal restriction sites. The recombinant pRT100 clones were designated as pRT100-APX. The CaMV35S-APX-poly(A) cassette in pRT100-APX was excised using PstI restriction enzyme. Both the HindIII restriction fragments of cry1Ac cassette (CaMV35S-cry1Ac-poly(A) and APX cassette CaMV35S-APX-poly(A)) were ligated using DNA ligase and then sub-cloned at HindIII/ PstI site of pCAMBIA3300 vector containing bar gene as selectable marker. The resultant recombinant pCAMBIA3300-cry1Ac-APX (pCAMBIA3300-CaMV35S-cry1Ac-poly(A)-CaMV35S-APX-poly(A)) vector was mobilised into E.coli HB101 cells and were confirmed by HindIII and PstI restriction digestions. Later, recombinant pCAMBIA3300-cry1Ac-APX was mobilised into Agrobacterium (LBA4404) by tri-parental mating by utilizing helper plasmid PRK2013 (Ramesh et al. 2004).

Genetic transformation and generation of transgenic plants

Rice cultivar BPT 5204 seeds obtained from Indian Institute of Rice Research (IIRR), Hyderabad (India), were used for genetic transformation experiments. LBA4404 harbouring recombinant pCAMBIA3300 (pCAMBIA3300-cry1Ac-APX) vector was employed for Agrobacterium mediated genetic transformation. Scutellum-derived calli of BPT 5204 seeds were co-cultivated with AgrobacteriumLBA4404 culture with an OD of 0.8. For selection of (pCAMBIA-cry1Ac-APX) transformants, co-cultivated calli is allowed for two rounds of selection on medium with 8 mg/L phosphinothricin. Actively growing calli is selected and moved to the proliferation medium. After 10 days, the proliferated calli is transferred onto shooting medium then to rooting medium. About 40-50 days old putative transformants were tested for their tolerance to 0.25% basta.

Molecular analysis

PCR analysis of pCAMBIA-cry1Ac-APX transformants

Genomic DNA was isolated from pCAMBIA-cry1Ac-APX putative transformants as well as from untransformed control (UC) plants as per McCouch (1988). PCR analyses was done utilizing cry1Ac gene
specific forward 5'-AGTCCAATCGAAAGTGTGG -3' and reverse 5'-AAAATAGCCGCATTGACACC-3' primers and APX gene specific forward 5'-ATCAGCTTGCTGGAGTGGTT-3' and reverse 5'-GCCGTTGAACCATCTGATT-3' primers the PCR products have been further analysed on a 1.0% agarose gel. DNA from the untransformed plants acted as negative control and plasmid DNA as positive control.

**Southern blot analysis of transgenic plants**

Southern blot analysis was performed using 18 μg of genomic DNA, isolated from pCAMBIA-cry1Ac-APX transgenic plants, digested with HindIII restriction enzyme. The DNA which has been digested was extracted on 0.8% agarose gel. Late after the denaturation and also neutralization, the DNA has been shifted to a H-N+ membrane (Amersham Pharmacia) and later cross-linking was performed through exposing to UV (1200 µJ for 60 s). The cry1Ac region and APX coding regions were radiolabeled with α-32P dCTP using ready to Go DNA labeling beads and used as a probe. After labelling, hybridization steps and washing of the membranes were carried out as per the instructions given by the manufacturer.

**Generation of homozygous transgenic lines**

To generate homozygous lines of pCAMBIA-cry1Ac-APX transgenic lines seeds obtained via putative transformants were put to growth on MS basal medium along with 6 mg/L phosphinothricin (PPT). After 10 days, the resistant (T2) seedlings were moved to pots and grown. The seeds obtained from T2 plants were later germinated on the MS medium containing 6 mg/L PPT for selection of homozygous lines.

**Functional validation of transgenic plants for Abiotic Stresses**

**Drought stress at seed germination and seedling stage**

Seeds of homozygous lines were surface sterilized with 70 % (v/v) ethanol for 1 min followed by 0.1% (w/v) Mercuric chloride (HgCl2) for 4 min and then washed with sterile distilled water for three times. To assess the drought stress response, the sterilized seeds were dried on sterile filter papers and transferred onto MS basal medium supplemented with 250 mM mannitol. The seed germination rates were taken late after 10 days. For stress treatment at seedlings stage, seeds of homozygous lines were germinated in Hoagland solution at 30°C for 2 week. Later they have been transferred to fresh Hoagland solution containing 250mM mannitol for 7 days. After stress treatment, the seedlings have been shifted to fresh Hoagland solution for recovery. After 7 days, the survival rate, shoot and root length, and seedlings biomass have been registered.

**Drought stress at the vegetative stage**

Seeds of homozygous lines were grown on MS medium and then established in pots. 40-50 days old plants in pots were selected for drought stress treatment. Drought (with-hold of water) stress treatment was applied at the vegetative stage for 10 days at30°C. After 10 days of treatment, plants were supplied with water at regular basis for recovery. Data on antioxidants, chlorophyll, water content, reducing sugars, proline content was deduced.
Drought stress at the reproductive stage

Plants are allowed to grow under normal conditions till the inflorescence. At reproductive stage, drought stress treatment was imposed by withholding water for 10 days at 30°C. After 10 days of withholding water, the plants were supplied with water for recovery. The data on number of filled grains per panicle, total chlorophyll content, anti-oxidants activity were recorded. Five plants were used in each treatment and all the experiments were repeated thrice.

Total chlorophyll content

Total chlorophyll content is calculated as per Lichtenthaler and Wellburn. Twenty five mg of fresh leaf of treated and untreated samples of transgenic plants along with UCare incubated in 10 ml of 80% acetone and left in dark for about 48 hours. Later the absorbance rate has been calculated at 663 and 647 nm for chl a and chl b content, respectively.

Proline content

This is carried out as per Bates (1973) documentation. One gram of fresh leaf from both transgenic, UC plants were broken down with 20 ml of 3% sulfosalicylic acid (W/V) and the obtained homogenate has been centrifuged for around 10 minutes at 10,000 rpm. The supernatant is utilized for proline estimation. Reaction mixture with 2.0 ml of glacial acetic acid, ninhydrin reagent and supernatant was boiled to 110°C for about 1 hour. Late after the reaction mixture is cooled down, 4 ml of toulene was included and vortexed for 30 about seconds over a cyclomixer. The chromophore from aqueous phase has been collected, absorbance is measured 520 nm. The concentration of Proline was analysed from the standard curve and given in microgram of proline per gram of the fresh material.

Estimation of reducing sugars

Leaf tissues (100 mg) of transgenic and UC plants were freezed using liquid nitrogen and made into a powder. Reducing sugars of about 2 times were separated from the powder by using 80% ethanol at about 95°C. The supernatant is concentrated by drying at about 80°C for around 2 hours. The residue was later dissolved in distilled water and the reducing sugars are used for estimating as per the available method (Miller 1959).

Extraction and estimation of antioxidant enzymes

The extraction procedure for both SOD and CAT is similar. Transgenic and UC plants leaf samples of 100mg were frozen by utilizing liquid N2 in order to avert proteolytic activity. The frozen tissues are broken down by utilizing 5ml extraction buffer with 100mM phosphate buffer (pH 7.5) and 0.5 mM EDTA and later centrifuged for about 15min at 12,000 rpm and 4°C. The supernatant was utilized used for the analysis of enzyme.

Estimation of Superoxide dismutase activity (SOD)
The activity of SOD was assessed by checking over the reticence of photochemical reduction of nitroblue tetrazolium. Later enzyme activity have been analysed by including 100μl of enzyme extract to 3ml of the reaction mixture having 13mM methionine, 2μM riboflavin, 50mM potassium phosphate buffer (pH 7.8), 75μM NBT, and 0.1mM EDTA. After that thereaction mixture was illuminated with 5000lux light intensity. Later absorbance was checked at 560 nm by utilizing spectrophotometer. SOD activity is the amount of enzyme required to impede half rate of inhibition of the NBT reduction.

**Catalase (CAT) activity**

Leaf samples from stressed plants were collected and homogenized in 50mM phosphate buffer (pH7.0). The obtained homogenate was later centrifuged at 8000g, 20min, 4°C. The extract of Enzyme is added to the H$_2$O$_2$-phosphate buffer (pH 7.0), and time taken for the absorbance at about 240nm from about 0.45-0.40 was considered. The Catalase activity was analysed as per Aebi (1984) and protein estimation was carried out using Bradford reagent.

**Malondialdehyde (MDA) content**

The leaf tissues were allowed for homogenization in 5ml 0.1% TCA (trichloroacetic acid) and then sent for centrifugation at 5000 g, 10min. 500μl of the obtained supernatant is mixed with 4ml of 20% TCA having thiobarbituric acid (TBA) (0.5%). Later, mixture is heated up at around 95°C, 30min, and then cooled down on ice, later centrifuged at 5000 g, 15min, and then the absorbance was noted at 532 and 600nm. After excluding the non-specific absorbance, MDA activity was measured by utilizing the extinction coefficient of 155mM$^{-1}$ cm$^{-1}$.

**Relative water content measurement**

To measure this, the leaves of transgenic and UC plants fresh weight was measured in relation to fresh leaf weight. Plants leaves were left at room temperature till no loss in weight (desiccated weight). Later after, the leaves were allowed to dry for about 24 hours, 70°C and the dry weights have been noted. The below is the formula used to measure RWC.

\[
\text{RWC (%) = } \frac{\text{desiccation weight - dry weight}}{\text{fresh weight - dry weight}} \times 100.
\]

**Quantitative Real Time PCR (qRT-PCR)**

For qRT-PCR analysis, RNA samples extracted from untransformed control(UC) and APX transgenic seedlings subjected to 200mM mannitol treatment for 24h along with unstressed plants were utilized for synthesizing first strand cDNA.qRT-PCR analyses were carried out using SYBR green master mix with Applied Biosystems 7500 real time PCR system programmed with 94°C (1min), 59°C (45 sec) and 72°C (1min) for 30 cycles. The RT-PCR products obtained were analysed through the melt curve analysis to check the specificity of PCR amplification. All the reactions were performed thrice and the relative expression ratio was calculated using $2^{-\Delta\Delta ct}$ method employing actin gene as a reference. The primers used for qRT-PCR were tabulated in supplementary material (Table S1).
Statistical analysis

All of the obtained data were determined by utilizing Student’s t-test for statistical significance. **P <0.01 and *P <0.05 shows differences at 1%, 5% level of significance, when compared with wild-type of control.

Results

Generation of p cry1Ac-APX transgenic plants

Agrobacterium harbouring p cry1Ac-APX was employed for development of transgenic plants in BPT 5204. Four transformates were obtained from a total of 3247 infected calli employing p cry1Ac-APX vector. Calli selected on phosphinothricin containing medium was utilized for the regeneration of transgenic plants (Fig. 1).

PCR and Southern blot analyses of p cry1Ac-APX primary (T₀) transformants

PCR analysis of p cry1 Ac-APX primary (T₀) transformants, employing cry1 Acforward and cry1 Aceverse primers, disclosed a 915 bp amplicon corresponding to cry1 Ac region. In addition, PCR analysis, employing APX forward and APX reverse primers, revealed the presence of 367 bp amplification band representing ascorbate peroxidase region of p cry1 Ac-APX vector (Fig. 2), Whereas UC failed to reproduce such amplicon.

Genomic DNA of p cry1 Ac-APX transgenic rice lines was digested with Hind III and Pst I restriction enzymes. Southern blot analyses was performed and probed with APX coding regions, displayed a hybridization signals at ~1.8 kb regions and such signals was not developed by untransformed control (UC) plant DNA. PCR and Southern blot analyses positive lines were selected for further analyses (Fig. 3).

Homozygous transgenic lines

T₂ seeds collected from the T₁ mature p cry1 Ac-APX plants, when germinated on MS basal medium containing PPT (4 mg/L) showed 100% germination, whereas heterozygous lines failed to show 100% germination.

Drought stress at germination stage

Drought stress tolerance of p cry1 Ac-APX transgenic lines were evaluated by germinating the seeds of homozygous lines on medium supplemented with 200mM mannitol. After 2 weeks of incubation, the transgenic plants (APX1, APX 2, APX 3 and APX4) showed higher germination rates of 80.35 ± 5.68, 82.67 ± 4.12, 81.41 ± 2.47 and 80.85 ± 5.31%, respectively, than UC (24.51 ± 3.54%) plants (Fig. 4).

Drought stress at seedling stage

Twenty one days old seedlings of p cry1 Ac-APX transgenic lines when subjected to 200mM mannitol stress showed higher survival rate, biomass, shoot and root length compared to UC plants. The survival
rates of pcry1Ac-APX transgenic lines (APX1, APX 2, APX 3 and APX4) disclosed higher survival rates of 84.48 ± 0.19, 89.04 ± 0.32, 86.38 ± 0.45 and 88.76 ± 0.28% under 200mM mannitol stress than UC plants (34.13 ± 0.05%). Transgenic and control seeds showed 100% survival rate under normal conditions. Similarly, the biomass of APX1, APX2, APX3 and APX4 transgenic lines under 200 mM mannitol stress recorded (108.24 ± 1.75, 114.47 ± 1.48, 110.21 ± 1.81 and 111.65 ± 1.05 mg FW) was significantly greater in comparison to UC (56.82 ± 1.18 mg FW) plants.

Moreover, the transgenic plants (APX1, APX 2, APX3 and APX4) showed greater shoot lengths of 7.14± 0.11, 7.59± 0.02, 7.27± 0.74 and 7.11± 0.33 cm, respectively, in mannitol (200mM) stress compared to UC (2.48± 0.09 cm) plants. Further, increased root lengths were produced by transgenic plants (6.75± 0.31, 6.84 ± 0.26, 6.25± 0.17 and 6.81± 0.13 cm) subjected to 200 Mm mannitol stress than that of UC (2.19± 0.14) plants (Fig. 5).

**Stress treatments at vegetative stage**

The pcry1Ac-APX transgenic plants (APX1, APX2, APX3 and APX4) along with control plants (50–60 days old) were subjected to drought (withholding water) stress for 2 weeks at 30°C. Then plants were allowed to grow to maturity under normal conditions (30°C). Transgenic plants when subjected to drought stress could reach the reproductive stage and set seeds whereas untransformed control plants failed to reach the reproductive stage (Fig. 6).

**Stress treatments at reproductive stage**

Transgenic (APX1, APX2, APX3 and APX4) plants along with control plants (85-95 days old) were subjected to drought stress for 2 weeks and then the plants were allowed to grow to maturity under normal conditions at 30°C. Increased panicle length was recorded in APX1, APX2, APX3 and APX4 transgenic plants (16.87 ± 0.19, 15.32 ± 0.27, 15.91 ± 0.31 and 15.02 ± 0.99 cm), respectively, than UC (9.25 ±0.42 cm) plants.

Moreover, enhanced average yield per plant (507.21 ± 5.67, 425.33 ± 9.58, 479.84 ± 9.21 and 492.68 ± 8.29 grains) was shown by APX1, APX2, APX3 and APX4 transgenic plants, respectively, whereas UC plants produced 196.54 ± 2.68 grains under similar stress conditions. Under normal conditions, there was no significant difference observed in panicle size and yield between control and pcry1Ac-APX transgenic plants (Fig. 7).

**Biochemical assays**

**Chlorophyll content**

After 2 weeks of drought stress treatment, the chlorophyll contents were estimated in control and pcry1Ac-APX transgenic plants. The chlorophyll contents of transgenic (APX1, APX2, APX3 and APX4) plants (2.19 ± 0.034, 2.28 ± 0.029, 2.17 ± 0.045 and 2.31 ± 0.067 mg g⁻¹ FW) was much higher compared
to UC (1.31 ± 0.017 mg g\(^{-1}\) FW) plants under drought stress. Under normal conditions, no significant
difference was observed in chlorophyll contents between control and transgenic plants (Fig. 8).

**Proline content**

Physiological basis of stress tolerance was understood by estimating the proline content under normal
and stress conditions. Under normal conditions, there is no significant difference was observed between
control and pcry1Ac-APX transgenic plants. Higher amounts of proline were accumulated in APX1, APX2,
APX3 and APX4 transgenic plants (658.71 ± 8.68, 629.34 ± 5.49, 619.19 ± 6.15 and 631.83 ± 9.47 µg g\(^{-1}\)
FW) than that of control (195.46 ± 6.35 µg g\(^{-1}\) FW) plants under drought stress (Fig. 8).

**Reducing sugars**

It was recorded that there was no significant differences in reducing sugars between UC and pcry1Ac-APX
transgenic plants under normal conditions. Under drought stress, the mean reducing sugars in APX1,
APX2, APX3 and APX4 (1.96 ± 0.068, 1.67 ± 0.019, 1.73 ± 0.027 and 1.81 ± 0.021 µg g\(^{-1}\) FW) plants was
found to be increased compared to UC (0.65 ± 0.057 µg g\(^{-1}\) FW) plants (Fig. 9).

**Superoxide dismutase activity (SOD)**

SOD activity was much higher in pcry1Ac-APX transgenic plants compared to control plants under
drought stress conditions. SOD activity recorded in APX1, APX2, APX3 and APX4 (12.94 ± 0.02, 13.41 ±
0.38, 12.64 ± 0.08 and 12.32 ± 0.11 U/mg protein) was significantly higher than that of control (7.58 ±
0.67 U/mg protein) plants. Under normal conditions there is no much difference between control and
transgenic plants (Fig. 9).

**Catalase activity**

Untransformed control and pcry1Ac-APX transgenic plants when subjected to drought stress showed
significant differences in their catalase activity. Transgenic APX1, APX2, APX3 and APX4 plants showed
(2.076 ± 0.091, 2.158 ± 0.054, 1.962 ± 0.076 and 2.049 ± 0.011 U/min mg) enhanced activity when
compared to control (1.017 ± 0.024 U/min mg) plants (Fig. 9).

**MDA content**

MDA contents in transgenic plants was reduced when compared with control plants under drought stress.
The recorded MDA contents in transgenic APX1, APX2, APX3 and APX4 plants (22.24 ± 0.95, 20.96 ±
0.37, 23.07 ± 0.82 and 20.57 ± 0.64 nmol g\(^{-1}\)FW) was much lower under drought conditions compared to
control (31.15 ± 0.47 nmol g\(^{-1}\)FW) plants. There was no much differences between the transgenic and
control plants under normal conditions (Fig. 9).

**Relative water content**
Relative water contents (RWCs) in the leaves of transgenic plants along with control plants were measured after drought treatment. Water loss was observed in both control and transgenic plants, the measured final relative water content of APX1, APX2, APX3 and APX4 (78.25 ± 1.62, 83.57 ± 2.14, 80.89 ± 1.87 and 79.52 ± 1.63%) plants was significantly higher than that of untransformed control (42.91 ± 1.24%) plants (Fig. 10).

RT-PCR analysis

To analyse the mRNA expression levels, quantitative RT PCR analysis was carried for selected stress responsive genes, viz., OsDREB, OsMYB and bZIP under stressed and unstressed conditions. The results showed an increase in the expression levels of OsDREB, OsMYB and bZIP in transgenic plants compared to the control under stress conditions (Fig. 11).

Discussion

Rice is the staple food for one third of world population, providing upto 80% of their daily diet. Being sessile, rice plants are being exposed to different adverse climatic stresses. Among all the abiotic stresses, drought is the most threatening one causing growth retardation and yield loss in rice plants. Drought stress causes more than 50% of agricultural yield loss across the world (You et al. 2014). To overcome and withstand the abiotic and biotic stresses, plants are being adopted to various physiological mechanisms. To maintain the supply of food to rising population, it is highly required to grow transgenic rice with enhanced yield even under drought conditions. Many transgenic approaches have been employed, among them overexpression studies have been proved to be the one of the best approach for developing high yielding rice varieties.

In the present study, pcry1Ac-APX vector was employed to generate overexpression lines in BPT 5204 rice variety. Cry1AC gene was isolated from Bacillus thuringiensis using full length primers and cloned into pRT100 vector. The cry1AC gene cassette was excised using HindIII restriction digestion. Moreover, ascorbate peroxidase (APX) gene was isolated from the rice cDNA and cloned into pRT100. APX gene cassette was excised using PstI enzyme and ligated with the cry1AC gene cassette and finally cloned into pCAMBIA3300 vector at HindIII/PstI site. The recombinant clones digested with HindIII and PstI restriction enzymes, released ~ 2.2 kb and ~ 1.8 kb DNA fragment corresponding to cry1AC and APX gene cassettes, respectively. These results represents the successful cloning of cry1AC and APX expression cassettes into pCAMBIA3300. The recombinant pcry1Ac-APX vector was stabilized onto Agrobacterium LBA4404 by the method of freeze-thaw.

Embryogenic calli produced from scutellum of Samba Mahsuri (SM) rice was co-cultivated with Agrobacterium LBA4404 harbouring pcry1 Ac-APX vector. A total of four putative pcry1Ac-APX transgenic plants were developed and confirmed by basta leaf dip assay, PCR and Southern blot analyses. During seed germination under drought stress (200 mM mannitol) the transgenic line CA-2 (82%) and CA-3 (81%) showed 3.4 and 3.3 times higher germination rates than UC (24%) plants. Similarly, in Arabidopsis, overexpression of OsRH58 affects the seed germination by marginal increasing the expression of
phytochrome B (PHYB) and lowering the levels of seed storage protein and LEA protein under drought stress conditions (Nawaz and Kang 2019). Moreover, in a study on rice, OsPM1 expression under drought stress was controlled by the AREB/ABF family transcription factor (OsbZIP46) and proved to be involved in ABA-regulated rice seed germination (Yao et al. 2018).

Likewise, under drought stress during seedling stage, CA-2 and CA-4 showed 2.61 and 2.58 times more survival rate than UC plants. A study on overexpression of OsLG3 in rice, showed an improved survival rates of 48–64% compared to wild type (17–28%) seedlings under severe drought stress for 7 days (Xiong et al. 2017). Further, under drought stress, pcry1 Ac-APX transgenic lines, CA-2 and CA-4, exhibited 2.03 and 1.98 times increased biomass compared to UC plants. Likewise, overexpression of Arabidopsis SHN1 in wheat exhibited improved recovery, lowered the stomatal density, minimized leaf water loss and accumulation of alkanes in the leaves lead to enhanced biomass production in transgenic plants (Bi et al. 2017).

In drought stress conditions, the transgenic lines CA-2 and CA-4 disclosed maximum root lengths of 3.12 and 3.10 times higher than UC plants. Overexpression of GmWRKY16 in Arabidopsis improved the seed germination rate, showed more than 75% survival rate and root length by regulating signaling genes (ABI1, ABI2, ABI4, and ABI5), stress-related marker genes (KIN1, LEA14, LEA76, and CER3), responsive genes (RD29A, COR15A, COR15B, and RD22) and ABA biosynthesis gene (NCED3) in drought (Xia et al. 2018). Likewise, in a study on Arabidopsis, heterologous overexpression of OsSADR1 improved germination, root and shoot lengths under 200 mM mannitol stress (Park et al. 2018).

PYrabactin resistance 1 Like/Regulatory Components of ABA Receptors (OsPYL/RCAR5) overexpression lines exhibited enhanced drought tolerance, survival rate, increased root and shoot length in drought stress in rice at vegetative stage (Kim et al. 2018). In an early report, rice SNAC1, binds to NACR of the OsERD1 promoter, conferring raised drought and salt resistance under vegetative stages (Hu et al. 2006). Moreover, overexpression of OsNAC14 in rice under drought stress, interacts with OsRAD51A1 promoter and recruits factors for DNA damage repair responses relating to tolerance to drought at vegetative stage (Shim et al. 2018). Likewise, constitutive overexpression of OsFTL10 bestowed drought in transgenic plants by up-regulation of OsMADS15 gene and regulating the expression of Ehd1 and OsMADS51 genes by feedback inhibition (Fang et al. 2019).

The transgenic CA-4 (2.31 ± 0.067 mg g⁻¹ FW) and CA-2 (2.28 ± 0.029 mg g⁻¹ FW) plants exhibited 3.78 and 3.73 times more chlorophyll content, in drought stress as compared to UC (0.61 ± 0.017 mg g⁻¹ FW) plants. The enhanced drought tolerance was believed to prevent the damage and maintain chlorophyll content (Kim et al. 2014). Correspondingly, overexpression of OsRab7 showed enhanced chlorophyll content and improved drought tolerance by regulating photosynthesis, gas-exchange attributes, antioxidant machinery, osmolytes that are involved in defence pathways (El and Alayafi 2019). Likewise, expression of AhCuZnSOD in tobacco plants deliberated improved tolerance to drought through seed germination and greater chlorophyll content that control plants (Xie et al. 2019).
Higher proline content was exhibited by pcry1Ac-APX transgenic CA-1 (658.71 ± 8.68 µg g⁻¹ FW) and CA-4 (631.83 ± 9.47 µg g⁻¹ FW) plants. Proline content in transgenic plants showed 3.37 and 3.23 times more than that of UC plants. The higher proline content in transgenic plants mediates as a stabilizer to aid the cells from abiotic stress (Ben et al. 2012). Proline gets accumulated under drought and acts as osmolyte, scavenging free radicals, and stabilizes the sub-cellular structures (Ashraf and Foolad 2007). Similarly, OsMYB6-overexpression plants evaluated by higher CAT, SOD and proline content and with lowered MDA content, exhibited enhanced tolerance to drought under drought stress (Tang et al. 2019).

In one of the studies, under drought stress, higher rates of photosynthesis, membrane stability, low electrolyte leakage and higher antioxidant activities were exhibited by ZmNF-YB16 overexpression lines suggesting its role in gene regulation included in photosynthesis and cellular antioxidants (Wang et al. 2018). The SOD activity was higher in transgenic line CA-2 and CA-1. It was recorded CA-2 and CA-1 exhibited 1.76 and 1.70 times higher SOD activity than UC plants. It was reported that overexpression of APX and Cu/ZnSOD in chloroplasts of sweet potato enhanced the drought tolerance, photosynthetic activity and recovery under drought stress (Lu et al. 2010). Plants to drought stress induce a very complex and effective strategies that includes stomatal closure to reduce plant transpiration and water loss, cell membrane stability is maintained by increasing SOD, CAT and other antioxidants and osmotic balance in cells is maintained by accumulating osmolytes (Ouyang et al. 2010).

The catalase activity was recorded maximum in transgenic CA-2 and CA-1. The activity was increased by 2.12 and 2.04 times in comparison to UC plants. A study on overexpression of ArabidopsisZAT18 (a C₂H₂ zinc finger protein) showed lower H₂O₂, more water content and greater activity of CAT under drought stress suggesting the involvement of ZAT18 in ROS scavenging (Yin et al. 2017). Under drought stress, CAT is believed to detoxify H₂O₂ to convert to H₂O and O₂ indicating improved CAT activity increases tolerance level to drought (Nahar et al. 2018).

MDA content in transgenic CA-4 and CA-2 was 33.96 and 32.71% less compared to UC plants in drought stress. OsLG3 over expression in rice lead to less accumulation of H₂O₂ and MDA in transgenic leaves suggesting the OsLG3 plants developed effective ROS scavenging system and reduced the lipid peroxidation by lowering the MDA levels (Xiong et al 2018). In addition, abiotic stress lead to lipid peroxidation, causing MDA accumulation (Mellacheruvu et al. 2016). Moreover, co-overexpression of OsGS1:1/OsGS2 in rice showed accumulation of proline, lower electrolyte leakage and malondialdehyde content in transgenic plants suggesting its involvement in tolerance and agronomic functions in stress conditions (James et al. 2018).

The water content in leaves of CA-2 and CA-3 showed 1.94 and 1.88 times higher than UC plants. The sugarcane R2R3-MYB genes, ScMYBAS1-2 and ScMYBAS1-3, expressed in rice, showed higher relative water content in transgenic lines than wild type under drought (Peixoto et al. 2018). Further, phenotype analysis of transgenic Arabidopsis lines overexpressing maize ZmASR3 gene showed greater water content in the leaves and less MDA levels, demonstrating that ZmASR3 in conferring tolerance to drought (Liang et al. 2019). TaNAC2 overexpression in rice rendered drought tolerance, abiotic stress response...
(OsLEA3, OsDREB2A, OsDREB1A, OsP5CS, SNAC1, OsCATA) genes (Mao et al 2012). In addition, overexpression studies on Arabidopsis promoted Drought Tolerance 1/HOMEODOMAIN GLABROUS11 gene in rice showed grain yield in drought (Yu et al. 2013). Moreover, in another study, OsRab7 gene raises grain yield and improves heat and drought tolerance in transgenic types by abiotic stress-responsive genesomolytes, and antioxidants.

**Conclusion**

The results achieved in the study suggests that the pcam-cry1Ac-APX transgenic lines showed improved tolerance to drought stress. Hence these transgenics are promising lines for enhanced yield compared to UC.

**Declarations**

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**Conflicts of interest:** The authors declare that they have no conflicts of interest.

**Author contribution:** NJ give the ideology and continuous supervision of the work. VD collected the methodology, samples, performed laboratory experiments, and achievement of results and writing of the manuscript.

**Ethics approval:** The finding in the study doesn't need any ethical approval

**Consent for publication:** It is to declare that all authors agreed with the content and that all gave explicit consent to submit and that they obtained consent from the responsible authorities at the institute/organization where the work has been carried out, before the work is submitted.

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**Figures**

![Figure 1](image1)

**Figure 1**

Selection and regeneration of APX putative transformants. (a) Selection of transformed calli on medium containing 5mg/l PPT (b) Regeneration of APX plantlets on regeneration medium
Figure 2

PCR analysis of APX putative transformants. (a) PCR analysis using cry1AC forward and reverse primers (b) PCR analysis performed with APX forward and reverse primers.

Figure 3

Southern blot analysis of APX putative transformants. Genomic DNA digestion with HindIII and PstI restriction enzymes and hybridized with APX coding sequence.
Figure 4

Germination of APX and UC seed on MS medium containing 200mM mannitol
Figure 5

Drought stress (200mM mannitol) treatment of APX and UC seedlings. Plant survival, total biomass, shoot length and root length of control and pcry1Ac-APX transgenic lines of rice subjected to well-watered and mannitol (250mM) stress
Figure 6

Drought stress treatment of transgenic APX and UC plants at vegetative stage

Figure 7
Graphical representation of panicle size and yield of transgenic APX and UC plants at reproductive stage

**Figure 8**

Chlorophyll and proline content of transgenic APX and UC plants with and without drought stress

**Figure 9**

Reducing sugars and antioxidant (SOS, catalase and MDM) assays of APX and UC plants with and without stress
Figure 10

Relative water content of APX and UC plants with and without stress
Figure 11

Relative expression levels of drought related genes

Supplementary Files

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