Evaluation of osmotic stress tolerance in transgenic Arabidopsis plants expressing Solanum tuberosum D200 gene

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ABSTRACT

Efficient stress-tolerant crops through genetic engineering remains one of the major challenges for plant biologists. Approximately 20–40% of genes of the known eukaryotic genomes, encode proteins of unknown function which lack currently defined motifs or domains. In a previous study, large-scale yeast functional screening approach in potato was used and 69 genes were reported to have hyperosmotic stress tolerance of yeast. Twelve out of 69 genes were found to have stress tolerance against multiple stresses. One of those 12 identified genes (StD200) encodes a protein of unknown function. In this study, we evaluated the tolerance against PEG-induced osmotic stress in transgenic Arabidopsis plants expressing putative abiotic stress-associated D200 gene. The D200 plants exhibited higher accumulation of chlorophyll and proline and reduced levels of oxidants compared to the wild-type (WT) control plants when subjected to PEG-induced osmotic stress conditions. Our quantitative Real-Time PCR results also suggested an increased accumulation of mRNA transcripts of genes encoding three major antioxidant enzymes in PEG-treated D200 plants compared to WT. Furthermore, improved photosynthetic parameters, Fv/Fm and performance index in PEG-treated D200 plants indicated that potato D200 gene is a potential candidate gene for developing stress-tolerant crops in future.

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Introduction

Plants are sessile organisms that are exposed to various adverse environmental stresses like salinity, heavy metal toxicity, heat/cold, and water deficit. The harmful effects induced by these stresses often result in agronomic crop yield losses. The underlying physiological and biochemical mechanisms of abiotic stress tolerance have been studied extensively over the years (Upadhyaya et al. 2011; Kumar et al. 2012; Gururani, Ganesan, et al. 2015; Gururani, Venkatesh, Ganesan, et al. 2015; Mickelbart et al. 2015). Transgenic plants have been generated by exploiting various genes and these plants tolerated various stresses via a combination of different mechanisms. With the onset of abiotic stress, energized electrons are allocated to dioxygen (O2) which is then used in two vital photosynthetic reactions, photorespiration and the Mehler peroxidase reaction. Later, this O2 is reduced to superoxide and hydrogen peroxide both of which are commonly categorized as reactive oxygen species (ROS) molecules (Gururani, Mohanta, et al. 2015; Gururani, Venkatesh, et al. 2015). Mechanism of intracellular ROS production during abiotic stress conditions and their detoxification have been studied extensively, but the precise mechanisms underlying the distribution of ROS in specific cellular compartments remain unclear (Gururani, Venkatesh, et al. 2015). Production of H2O2 has been attributed to the chloroplasts and peroxisomes (Noctor et al. 2014). The production of ROS in leaf tissues is regulated by the harvesting and distribution of light energy to the photosynthetic machinery (Tikkkanen et al. 2010). The damage to DNA, lipids, and proteins caused by ROS molecules is known to be the result of oxidative stress (Foyer & Shigeoka 2011). The initial effects of ROS accumulation in chloroplasts include the damage to photosystem II (PSII) D1 protein (Gururani, Venkatesh, et al. 2015; Gururani et al. 2017; Sasi et al. 2018), suppression of ROS-responsive chloroplastic enzymes (Kato & Sakamoto 2009), and disintegration of thylakoids (Gradiao et al. 2009; Gururani, Venkatesh, et al. 2015). To ensure survival under the adverse environmental conditions, plants have developed efficient antioxidant machinery that primarily has two arms, (i) enzymatic components like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR) and; (ii) non-enzymatic antioxidants like ascorbic acid (AA), reduced glutathione (GSH), α-tocopherol, carotenoids, flavonoids, and the osmolyte proline (Gill & Tuteja 2010). These two components work hand in hand to scavenge ROS (Das & Roychoudhury 2014). When a plant is exposed to stressful conditions, a wide array of stress-responsive genes are triggered that lead to increased or decreased accumulation of proteins. A number of these proteins have been identified and explored in the past in order to generate stress-tolerant transgenic crops. However, there are many such proteins that remain to be identified in terms of their function, particularly under stressful conditions. In a previous study by Kappachery et al. (2013), potential drought tolerance genes from...
Solanum tuberosum were identified using a large-scale yeast functional screening approach. The authors reported 69 genes associated with hypersomotic stress tolerance. In this study, we have cloned 1 of those 69 putative stress-tolerant genes named StD200 (hereafter D200) into Arabidopsis plants and evaluated the performance of transgenic lines under polyethylene glycol (PEG) induced osmotic stress.

Materials and methods

Plant material and growth conditions

Non-transgenic (NT) and T3 transgenic Arabidopsis lines Columbia (Col-0) ecotype expressing an S. tuberosum D200 gene (Genbank Acc. No. JX951423) encoding a hypothetical protein with unknown function was used for the study. The Arabidopsis D200 lines were generated by amplifying the D200 coding sequence (Kappachery et al. 2013) and cloned into the plant expression vector pMDC32. The cloned D200 gene was placed downstream of the CaMV 35S promoter and the orientation of cloned gene was confirmed by DNA sequencing. Binary vectors harboring the cloned gene were then transferred into Agrobacterium tumefaciens strain GV3101 by electroporation. The Arabidopsis D200 lines were generated by amplifying the gene (Genbank Acc. No. JX951423) and cloned into the plant expression vector pMDC32. The cloned D200 gene was placed downstream of the CaMV 35S promoter and the orientation of cloned gene was confirmed by DNA sequencing. Binary vectors harboring the cloned gene were then transferred into Agrobacterium tumefaciens strain GV3101 by electroporation. The Arabidopsis plants were transformed by the floral dip method as described earlier (Clough & Bent 1998).

Two-week-old wild type (WT) and T3 D200 transgenic Arabidopsis thaliana L. were used for the experiments. The seeds were surface sterilized with 5% sodium hypochlorite (v/v) for 15 min and rinsed 4–5 times with distilled water before transferring to half-strength Murashige and Skoog medium (7 g/l agar) with hygromycin (20 µg ml⁻¹). Plants were grown under controlled conditions in plant growth chambers at a photon flux density of 150 µmol m⁻² s⁻¹ (16:8 h day/night period) at a relative humidity of 60%, and a temperature of 22 ± 2°C.

Stress treatment and estimation of chlorophyll content

Two-week-old wild type (WT) and T3 D200 transgenic Arabidopsis plants that survived on the selection medium were selected for the stress assays. In order to induce PEG-induced osmotic stress in plants, an optimum concentration was determined in preliminary experimental analyses where different concentrations of PEG were tested in the in vitro grown Arabidopsis seedlings. While 5% PEG exhibited little effect in 7 days, 15% and 20% PEG showed significant damage on plant growth and development in 4–5 days. Hence, an optimum concentration of 10% PEG was selected for the stress assays. Three individual transgenic events namely D200-81, D200-82 and D200-85 as well as WT plants were allowed to grow in MS-medium containing 10% PEG in order to induce osmotic stress in the plants. Chlorophyll content from stressed and control samples was estimated as described earlier (Gururani, Venkatesh, Ganesan, et al. 2015).

Estimation of hydrogen peroxide and proline

Hydrogen peroxide (H₂O₂) content in leaves collected from the WT, D200 transgenic lines treated with PEG was determined as described earlier (Cheeseman 2006). A standard curve was prepared from serial dilutions (0–5 µM conc. of H₂O₂; R²=0.9403) to calculate the H₂O₂ concentrations. The data were normalized and expressed in µmol H₂O₂ g⁻¹ fresh weight (FW) of leaves.

Proline extraction and colorimetric estimation were carried out with leaf samples from WT, D200-81, D200-82 and D200-85 plants subjected to PEG-induced osmotic stress as described earlier (Gururani, Ganesan, et al. 2015). An approximately 500 mg leaf sample was ground and homogenized in 10 mL of 3% aqueous sulfosalicylic acid. Equal volumes (2 mL each) of the filtered homogenate, acid-ninhydrin, and glacial acetic acid were mixed and allowed to react for 1 h. The reaction was stopped by transferring the tubes on ice and finally, the chromophore-containing phase was extracted with 4 mL of toluene. The absorbance of extracted phase was recorded at 520 nm. Proline concentration was determined from a standard curve and calculated as µg/g DW.

Morphological analyses and growth parameters

The morphological characteristics and growth parameters were examined after growing the WT and D200 transgenic plants for 10 days on MS medium containing 10% PEG. Plant height, leaves/plant, flowers/plant, fresh weight and nodes/plant were measured after subjecting the plants to PEG-induced osmotic stress.

Quantitative real time PCR

After 10 days of PEG-induced osmotic stress, leaf samples were collected and stored at −80°C until further experiment. Total RNA from Arabidopsis rosette was isolated with the Norgen Biotek, Canada RNA isolation kit according to the manufacturer’s instructions. RNA samples were treated with DNAse I (Qiagen) for removal of DNA contamination. cDNA was synthesized using Norgen’sTruScript™ kit (Norgen Biotek, Canada) according to the manufacturer’s instructions. cDNA was diluted in sterile water (3:7) before using as a template for qRT-PCR analysis. qRT-PCR was performed using Mx3000P qPCR system (Agilent Technologies, USA) and LF TaqPCR SYBR Mix (Applied Biosystems, USA). Primers (Table 1) were designed using PRIMER3 program based on the following parameters: 23–27 nucleotides, Tm 60°C ± 3°C, product/amplicon size 200–250 bp. Prior to qRT-PCR, specificity of primers for each gene was checked by analyzing individual dissociation/melting curve. At1g13440 (GAPDH) encoding glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Threshold of 0.1 was set manually to obtain a threshold cycle (Ct) value for each gene. Ct values for all genes of interest (CLG0I) were normalized to the Ct values of GAPDH (Clt. GAPDH) for each replication [ΔCt = (Clt. GOI) – (Clt. GAPDH)] (Choi et al. 2017). Relative transcript levels of each gene were normalized to the Ct values of GAPDH (Clt. GAPDH) for each replication [ΔCt = (Clt. GOI) – (Clt. GAPDH)] (Choi et al. 2017). Relative transcript levels of each gene were

| Gene Name | Sequence |
|-----------|----------|
| StD200-F  | 5'-TCC CTG GGT CGA TCT GTA AA-3' (20mer) |
| StD200-R  | 5'-GAG CAT ATG CGG CAT TGG A3-3' (22mer) |
| At-APX-F  | 5'-TGCA GAG AAT AGG TCT GC-3' (20mer) |
| At-APX-R  | 5'-CCT TCC TTC TCT CGC TCT AA-3' (20mer) |
| At-CAT-F  | 5'-ATT TGC TTC ATC GGG AAG GA-3' (20mer) |
| At-CAT-R  | 5'-CTT CAA CAA AAC GGT TCA CGA-3' (20mer) |
| At-SOD-F  | 5'-TCC ATG CAG ACC CTG ATG AC-3' (20mer) |
| At-SOD-R  | 5'-CCT GGA GAC CAA TGA TGC C-3' (19mer) |
| At-GAPDH-F | 5'-GAG AGT TGG TGT GGT GAG TTC-3' (24mer) |
| At-GAPDH-R | 5'-GGT TTG AGT TAG CAC GAG GTA A-3' (24mer) |
calculated with respect to GAPDH (% relative expression to GAPDH) using $2^{-\Delta Ct}$ value $[2^{-\Delta Ct} \times 100]$ and plotted in graph. Mean values were obtained from three biological replicates, and the standard errors are indicated by error bars.

**Chlorophyll a (Chl a) fluorescence measurement**

Chlorophyll a fluorescence transients of dark-adapted intact leaves of the control (NT-Control and SIP-Control) and PEG-treated (NT-PEG and SIP-PEG) Arabidopsis plants were measured using a Pocket PEA (Plant Efficiency Analyzer, Hansatech, UK). Intact plants were kept in dark for at least 1 h before taking the measurements. The plotted fluorescence values were the averaged means of five measurements per plant made on marked leaves of three different plants of individual WT and D200 lines. The maximal fluorescence ($F_{m}$) and the minimal fluorescence ($F_{0}$) of sampled leaves were used to calculate the $F_{v}/F_{m}$ ratio. The parameter $F_{v}/F_{m}$ represents the maximum quantum yield of PSII if all the PSII centers were open (Gururani, Upadhyaya, Baskar, et al. 2013). Therefore, $F_{v}/F_{m}$ indicates the intactness of the Light Harvesting Complex (PSII/LHC) unit and the probability of a trapped photon within the reaction center to cause a photochemical event. The optimal value of $F_{v}/F_{m}$ varies between a range of 0.79 and 0.83 for most plant species, and lower values indicate that the plant is lacking an optimal health state. Similarly, performance index (PI) is calculated from the recorded data which is the sum of overall expressions that represent a kind of internal force of the sample to resist stress thereby indicating the vitality of the samples used (Gururani et al. 2012; Choi et al. 2017; Ghosh et al. 2017; Gururani et al. 2017).

**Statistical analysis**

At least three replicates were used in all experiments and the experiments were repeated three times. Data was analyzed using the Origin 8.1 software program and the statistical differences were determined using one-way ANOVA followed by Tukey’s multiple comparison tests, and standard error was calculated using the n values of each experiment.

**Results**

**Phenotypic changes and validation through ROS and chlorophyll estimation**

WT and D200 transgenic plants exposed to PEG-induced osmotic stress conditions showed a significant increase in stress tolerance in D200 plants. The difference between WT and D200 plants treated with PEG was clearly visible, as the D200 plants appeared much greener and healthier than the WT plants (Figure 1). Chlorophyll estimation in WT and D200 plants further showed the difference in the physiological state of these plants. A significant difference (at $p \leq .05$) in the chlorophyll content of WT and D200 plants was observed after PEG-induced osmotic stress (Table 2). After 10 days of exposure to PEG-induced stress, D220-81, D200-82 and D200-85 plants exhibited 51.4%, 49.2% and 35.7% higher total chlorophyll content respectively compared to the WT plants. Similar increased levels of chlorophyll content in SIP transgenic Arabidopsis plants under PEG-induced stress has recently been reported (Gururani et al. 2017).

Accumulation of H$_2$O$_2$ in WT and D200 plants as a consequence of stress imposed by high levels of PEG was determined. Our results indicated that D200 plants accumulate less H$_2$O$_2$ as compared to WT plants under PEG-induced osmotic stress (Figure 2(a)). On average, the WT plants accumulated 54% higher levels of H$_2$O$_2$ than D200-81 and D200-82 and 88% higher than D200-85 plants. Similar reports where reduced H$_2$O$_2$ accumulation was observed in stress tolerant transgenic plants have been documented in the past (Lee et al. 2007; Gill & Tuteja 2010; Gururani, Upadhyaya, Strasser, et al. 2013; Ji et al. 2013). Proline is known as a common osmolyte in higher plants and accumulates in response to almost all known abiotic stresses. Accumulation of proline was measured in the WT, D200-81, D200-82 and D200-85 plants subjected to PEG-induced osmotic stress. A significantly higher amount of proline accumulated in D200 transgenics compared to WT in response to PEG-induced osmotic stress (Figure 2(b)). After 10 days of exposure to PEG-induced stress, D220-81, D200-82 and D200-85 plants exhibited 49.6%, 46.7% and 70.8% higher proline content, respectively compared to the WT plants.

**Table 2.** Chlorophyll estimation in corresponding leaf samples of wild-type Arabidopsis (WT) and Arabidopsis plants expressing D200 (D200-81, D200-82, D200-85) leaves after 10 days of PEG-induced osmotic stress.

| Sample       | ChlA (µg/mg FW) | ChlB (µg/mg FW) | Total Chl (µg/mg FW) |
|--------------|-----------------|-----------------|----------------------|
| WT           | 1.04 ± 0.08     | 0.35 ± 0.14     | 1.4 ± 0.09           |
| D200-81      | 1.64 ± 0.06     | 0.48 ± 0.08     | 2.12 ± 0.02          |
| D200-82      | 1.71 ± 0.08     | 0.30 ± 0.02     | 2.09 ± 0.09          |
| D200-85      | 1.22 ± 0.09     | 0.22 ± 0.14     | 1.9 ± 0.14           |

Values represent the mean ± S.E. from three independent assays with three replicates for each treatment. Different letters in each column indicate significant differences ($p \leq .05$) in chlorophyll content between treatments after Tukey’s test ($n = 3$).
Significant differences were observed between WT and D200 transgenic plants in terms of root and shoot lengths after exposing them to PEG-induced osmotic stress conditions on MS medium for 10 days (Figure 3). While D200 transgenic plants appeared greener with more root and shoot lengths, WT plants were shorter and showed symptoms of osmotic stress as their leaves appeared yellowish. Similarly, significant differences were recorded in terms of growth parameters of WT and D200 plants after 10 days of PEG-induced osmotic stress. Average plant height of D200-81, D200-82 and D200-85 was recorded 40%, 72.2% and 63.3% higher respectively compared to that of WT plants. In terms of root length, D200-81, D200-82 and D200-85 showed significantly increased root lengths (4.3, 4.8 and 4.3 cm) compared to that of WT plants (1.98 cm) under PEG-stress. However, there was no significant change in some of the growth parameters between WT and D200 plants. For instance, leaves/plant were recorded similarly in all the WT and D200 plants which ranged between 13.8 leaves/plant to 14.3 leaves/plant in D200 plants while WT plants showed an average of 14 leaves/plant (Table 3). Importantly, fresh weight per plant was recorded two times higher in D200 plants compared to that of WT plants. Finally, nodes/plant parameter showed varied difference among D200 plants ranging from 3.8 nodes/plant in D200-81 plants to 6.6 nodes/plant in D200-82 plants. Although the WT plants showed only 3.3 nodes/plant, this was not found significantly different to that of D200-81 and D200-85 in the statistical analyses (Table 3).

Expression analysis of StD200 under control and stressed conditions

WT and D200 transgenic lines were analyzed for the accumulation of StD200 mRNA transcripts under normal and PEG-induced osmotic stress. Since the WT plants do not express StD200 gene, mRNA expression was not detected in those...
plants. Variable expression of StD200 was recorded in D200-81, D200-82 and D200-85 lines under normal as well as PEG-induced osmotic stress conditions (Figure 4). The StD200 gene showed highest expression in D-200-85 lines followed by D200-81 and D-200-82 lines.

Expression analysis of genes encoding ROS-scavenging enzymes

qPCR was performed in WT and transgenic Arabidopsis lines for analyzing the expression levels of three antioxidant enzymes encoding genes, APX, CAT and SOD. The expression levels of all three genes in all three PEG-treated transgenic lines i.e. D200-81, D200-82 and D200-85 were recorded significantly higher. The expression levels of APX, CAT and SOD were recorded highest in transgenic line D200-82 under stress conditions (Figure 5) indicating that this line showed highest level of tolerance against PEG-induced osmotic stress. The high spontaneous expression of ROS scavenging enzymes gives the transgenic Arabidopsis sufficient protection against elevated level of radicals produced by abiotic stress.

Changes in maximum quantum yield of PSII (Fv/Fm) and performance index

The \( F_v/F_m \) values that express the maximum quantum yield of PSII (Gururani et al. 2012) were in the range of 0.83 (WT-C and D200-85-C) to 0.85 (D200-81-C) under non-stressed conditions and from 0.71 (WT-PEG) to 0.80 (D200-85-PEG) under PEG-induced osmotic stress conditions (Figure 6(a)). A sharper decline in \( F_v/F_m \) in WT subjected to PEG-induced osmotic stress compared to the D200 plants was noted, thus indicating that D200 plants sustained the stress conditions significantly better than the WT plants. This observation was further confirmed with the estimation of the performance index (PI), which is considered as an indicator of sample vitality. The PI values of D200 plants were significantly higher than that of the WT plants under PEG-induced osmotic stress conditions (Figure 6(b)). As expected, the PI of both WT and D200 plants under no stress control conditions remained similar ranging between 4.98 in WT-C samples and 5.14 in D200-81-C samples. However, a sharp decline in PI was observed in all plants after 10 days of PEG treatment. On average, the PI values of D200-81, D200-82 and D200-85 were recorded 60%, 38.4% and 23% higher respectively compared to that of WT plants in PEG-treated plant samples.

Discussion

Approximately 20–40% of all known eukaryotic genomes contain genes encoding proteins of unknown function (Golley et al. 2006). These proteins have been classified as proteins with obscure features (Luhua et al. 2008). While transcriptome and proteome studies indicate that many such proteins play critical roles in plants, it is imperative to carry out their functional characterization for the rapid development of stress-tolerant plant cultivars. In an earlier study,
Davletova (2005) reported several proteins of unknown function that responded to endogenous oxidative stress in Arabidopsis. Later, Luhua et al. (2008) constitutively expressed them in transgenic plants in order to reveal their putative role in oxidative stress tolerance. Oxidative stress as our source for genes of unknown function was intentionally selected because this stress is considered as a secondary stress to almost all abiotic stresses. Moreover, many of the known pathways and genes involved in the response of different organisms to oxidative stress have overlapping functions and/or structural similarities (Mittler et al. 2004; Gill and Tuteja 2010; Mittler et al. 2011).

Functional screening-based approach, using yeast or bacteria as an experimental system, has been efficiently used to identify genes with putative roles in the stress tolerance of plants (Tanaka et al. 2007; Ezawa & Tada 2009; Eswaran et al. 2010; Kumar et al. 2012). Recently, Kappachery et al. (2013) identified 69 potential drought tolerance genes in potato employing a yeast-based functional screen approach. A cDNA expression library of selected cDNAs that exhibited resistance against osmotic stress was constructed (Kappachery et al. 2013). Out of 69 identified genes, 61 were found to code for proteins of known functions while 8 were found to have unknown functions. Interestingly, one of the yeast transformants that expressed potato D200 protein of unknown function exhibited remarkably high to multiple stresses including drought, high temperature and high salinity. In this study, transgenic Arabidopsis plants expressing potato D200 gene were generated and evaluated for their performance under PEG-induced osmotic stress. After analyzing the D200 protein sequence, we found that this 136 amino acid residues protein has a molecular weight of approximately 14.8 kDa and a theoretical isoelectric point (pI) of 6.07 (https://web.expasy.org/cgi-bin/protparam). The pI of this protein indicates that it is most likely a cytoplasmic protein that is required for its function in the cytosol (Schwartz et al. 2001; Kiraga et al. 2007).

The D200 Arabidopsis plants accumulated higher amount of total chlorophyll content than the WT plants, when subjected to PEG-induced osmotic stress for ten days (Table 2). The plants appeared greener with visible differences in terms of growth between D200 and WT plants (Figure 1). H2O2 is a non-radical ROS, that acts as a signaling molecule when present at low concentrations and causes oxidative stress at high concentrations (Foyer & Shigeoka 2011). We found that the D200 plants accumulate less H2O2, as compared to WT plants under PEG-induced osmotic stress (Figure 2(a)). Similar results of reduced accumulation of H2O2 in stress-tolerant plants have been documented previously (Upadhyaya et al. 2011; Gururani, Upadhyaya, Baskar, et al. 2013; Gururani, Upadhyaya, Strasser, et al. 2013). Proline plays a pivotal role in stabilizing the subcellular structures and detoxifying the free radicals, therefore its accumulation is considered as an indicator of efficient abiotic stress tolerance in plants at metabolic level (Ashraf & Foolad 2007). We recorded a significantly higher proline accumulation in the D200 Arabidopsis plants under PEG-induced osmotic stress, relative to the WT plants (Figure 2(b)) demonstrating the requirement of proline in stress tolerance and integrity of the cellular system for survival. These observations corroborated with the differences between WT and D200 plants grown under osmotic stress conditions (Table 3). In particular, significantly higher plant height, flowers/plant fresh weight and root length in D200 plants compared to WT plants, suggested that the expression of potato D200 gene conferred notable osmotic stress tolerance in Arabidopsis plants. Interestingly in our qPCR analyses of StD200 in Arabidopsis transgenic lines, it was found that the expression levels of StD200 were highest in D200-85 lines followed by D200-81 and D200-82 lines under control conditions. A concomitant increase of StD200 expression levels in these lines was observed under PEG-induced osmotic stress conditions, suggesting that osmotic stress tolerance conferred in these plants was associated with the overexpression of StD200 in transgenic Arabidopsis lines.

Abiotic stresses lead to an increase in the formation of ROS at the cellular level. ROS molecules like H2O2 and hydroxyl radicals (OH) cause lipid peroxidation of membranes (Gill & Tuteja 2010). In addition to protecting the membranes and DNA from damage, ROS scavenging enzymes such as APX, CAT and SOD remove the free radicals produced during abiotic stress conditions. To determine the putative changes in the expression of genes encoding the ROS-scavenging enzymes, the WT and D200 plants were grown under PEG-induced osmotic stress conditions. The mRNA expression of SOD, CAT and APX in D200 plants growing under osmotic stress conditions increased considerably when compared with that in the WT plants (Figure 4). However, the expression levels of these genes reduced in PEG-treated WT samples compared

![Figure 6](image-url)
to non-stress control conditions in the same plants. This happened probably because the WT leaf samples had almost reached the stage of senescence after 10 days of PEG treatment. In contrast, the transgenic leaf samples were still green and significantly in better condition after the PEG treatment.

Photosynthesis is the most important process on earth that directly or indirectly gets affected by the adverse environmental conditions. PSII is considered the most vulnerable component of photosynthetic machinery that bears the brunt of stress conditions (Gururani, Venkatesh, et al. 2015). To assess the changes in the photosynthetic machinery of WT and D200 plants subjected to PEG-induced osmotic stress, we measured the photosynthetic efficiency of these plants, in terms of $F_v/F_m$, which indicates the maximum quantum yield of PSII. Abiotic stresses such are known to decrease the quantum efficiency of PSII photochemistry (Briantais et al. 1996; Gururani et al. 2017). Several reports suggest that measuring $F_v/F_m$ to examine the photosynthetic efficiency of different crop plants subjected to various stresses is an efficient tool in stress physiological studies (De Ronde et al. 2004; Yusuf et al. 2010; Mathur et al. 2011). The $F_v/F_m$ values were in the range of 0.71–0.85 in the WT and D200 plants under normal and PEG-induced osmotic stress conditions (Figure 6). Higher $F_v/F_m$ values in D200 plants under osmotic stress conditions compared to that of WT plants indicated a better optimal health of transgenic plant samples. Additionally, the PI of the plants, which indicates the sample vitality, was also recorded. The overall PI represents a single multi-parametric expression that combines all three independent functional steps of photosynthesis: the density of the reaction centers in the chlorophyll pool, trapped excitation energy, and its conversion to the electron transport (Strasser et al. 2000; Strasser & Tsimilli-Michael 2001). The PI values shown in Figure 6 clearly indicate that the photosynthetic performance of D200 plants under osmotic stress conditions significantly enhanced, indicating that these plants resisted the stressful conditions better than the WT plants.

In conclusion, the results shown here demonstrate that S. tuberosum D200 gene that encodes for a 14 kDa, 136 amino acid residue protein of unknown function is a potential candidate gene for the development of stress-tolerant plant cultivars. However, further characterization and proteomic studies are warranted in order to exploit the potential of this protein in stress physiological studies.

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SK, GBH, generated the D200 Arabidopsis transgenic lines, MAG and JV conceived and designed the experiments, MAG, SA, THH, SS conducted the experiments, MAG wrote the manuscript, MAG, SA, THH and SS analyzed the data and revised the manuscript.

Disclosure statement
No potential conflict of interest was reported by the authors.

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