Vegetative cell wall protein OsGP1 regulates cell wall mediated soda saline-alkali stress in rice (#84908)

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Vegetative cell wall protein OsGP1 regulates cell wall mediated soda saline-alkali stress in rice

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Plant growth and development are inhibited by the high levels of ions and pH due to soda saline-alkali soil, and the cell wall serves as a crucial barrier against external stresses in plant cells. Proteins in the cell wall play important roles in plant cell growth, morphogenesis, pathogen infection and environmental response. In the current study, the full-length coding sequence of the vegetative cell wall protein gene *OsGP1* was characterized from Lj11 (*Oryza sativa longjing11*), it contained 660 bp nucleotides encoding 219 amino acids. Protein-protein interaction network analysis revealed possible interaction between CESA1, TUBB8, and OsJ_01535 proteins, which are related to plant growth and cell wall synthesis. OsGP1 was found to be localized in the cell membrane and cell wall. Furthermore, overexpression of *OsGP1* leads to increase in plant height and fresh weight, showing enhanced resistance to saline-alkali stress. The ROS (reactive oxygen species) scavengers were regulated by OsGP1 protein, peroxidase and superoxide dismutase activities were significantly higher, while malondialdehyde was lower in the overexpression line. These results suggest that *OsGP1* may be involved in stress response pathway, by enhancing cell wall mediated saline-alkali stress tolerance in rice.

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Abstract

Plant growth and development are inhibited by the high levels of ions and pH due to soda saline-alkali soil, and the cell wall serves as a crucial barrier against external stresses in plant cells. Proteins in the cell wall play important roles in plant cell growth, morphogenesis, pathogen infection and environmental response. In the current study, the full-length coding sequence of the vegetative cell wall protein gene *OsGP1* was characterized from Lj11 (*Oryza sativa longjing11*), it contained 660 bp nucleotides encoding 219 amino acids. Protein-protein interaction network analysis revealed possible interaction between CESA1, TUBB8, and OsJ_01535 proteins, which are related to plant growth and cell wall synthesis. OsGP1 was found to be localized in the cell membrane and cell wall. Furthermore, overexpression of *OsGP1* leads to increase in plant height and fresh weight, showing enhanced resistance to saline-alkali stress. The ROS (reactive oxygen species) scavengers were regulated by OsGP1 protein, peroxidase and superoxide dismutase activities were significantly higher, while malondialdehyde was lower in the overexpression line. These results suggest that *OsGP1* may be involved in stress response pathway, by enhancing cell wall mediated saline-alkali stress tolerance in rice.

Introduction

The Songnen Plain of China is one of the three most concentrated saline-alkali lands in the world (*Li et al., 2003*), and it is an important reserve resource of cultivated land in China (*Jiang et al., 2019*). The salt in the soil of Songnen Plain mainly exists in the form of alkaline salts ($NaHCO_3$ and Na_2CO_3), with alarmingly high concentration of salt and pH. Rice is one of the four major

cereal crops (*Zhang et al., 2003*), the development of saline-alkali resistance in rice is an important research topic for improving the quality and efficiency of rice plantation and strengthening food security. Soda saline-alkali soil causes damage to plants mainly because of the high levels of Na^+, CO_3^{2-}, HCO_3^-, and extreme alkaline conditions (*Wang J et al., 2022*). Salt stress inhibits rice seed germination, seedling growth, and reproductive development (*Li et al., 2003*), leading to a decrease in leaf area, stalk, stem diameter, root activity, nutrient synthesis, accumulation, and transport, and interferes with young spike differentiation in reducing its effective spike number (*Wang Y et al., 2022*). Salt-alkali stress also leads to various physiological and molecular changes and hinders plant growth by inhibiting photosynthesis, thereby reducing available resources and inhibiting cell division and expansion. Plants have evolved many biochemical and molecular mechanisms to cope with saline-alkali stress (*Pastori and Foyer, 2002*), mainly including ion-selective absorption, accumulation of osmotic adjustment substances, and scavenging of reactive oxygen species (ROS) (*Liang et al., 2018*). Salt stress leads to a large influx of Na^+ into plant cells to induce ion toxicity, resulting in an imbalance of intracellular ion homeostasis, plants can promote osmotic balance at the cellular level through the synthesis of soluble sugars, proline, and other substances. ROS accumulates because of salt stress (*Vaidyanathan et al., 2003*), and plants scavenge excess ROS by producing superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and catalase (CAT) to avoid internal damage.

When plants are exposed to harsh climatic condition, the first to sense the stress is the cell wall. Plant cell walls surround cells and provide external protection and intercellular communication, and they are mainly composed of polysaccharides (cellulose, hemicelluloses, and pectins), polymers such as lignin, and a small amount of cell wall proteins (CWPs) (*Jamet and Dunand, 2020*). CWPs are divided into nine functional classes, including proteins acting on carbohydrates, oxidoreductases, proteases, proteins related to lipid metabolism, proteins possibly involved in signalling, proteins with predicted interaction domains, miscellaneous proteins, proteins of unknown function, and structural proteins (*Calderan-Rodrigues et al.,2019*). CWPs are major players in cell wall remodelling and signalling and play an important role in plant cell growth and development, morphogenesis, pathogen infection, and environmental response. FERONIA (FER), a plasma-membrane-localized receptor kinase from *Arabidopsis*, is necessary to sense the defects of the cell wall. Sensing of the salinity-induced wall defects is possibly a direct consequence of the physical interaction between the extracellular domain of FER and pectin. FER-dependent signalling elicits cell-specific calcium transients that maintain cell-wall integrity during salt stress (*Feng et al., 2018*). Leucine-rich repeat extensins (LRXs) are chimeric proteins in the cell wall. LRXs bind rapid alkalinization factor (RALF) peptide hormones that modify cell wall expansion and directly interact with the transmembrane receptor FER (*Herger et al., 2019*). RALF in turn interacts with FER. LRXs, RALFs, and FER function as a module to transduce cell wall signals to regulate plant growth and salt stress tolerance (*Zhao et al., 2018*).

Hydroxyproline-rich glycoproteins (HRGPs) are a superfamily of CWPs. According to the 'Hyp contiguity hypothesis', contiguous and non-contiguous clustered Hyp residues are the sites

attached by arabinoside and arabinogalactan polysaccharide, respectively (*Ma and Zhao, 2010*). HRGPs modification involves proline (Pro) hydroxylation and subsequent O-glycosylation on Hyp residues (*Hunt et al., 2017*). HRGPs consist of three members: hyperglycosylated arabinogalactan proteins (AGPs), moderately glycosylated extensins (EXTs), and lightly glycosylated proline-rich proteins (PRPs), which function in diverse aspects of plant growth and development (*Showalter et al., 2010*). A total of 162 HRGPs have been identified in *Arabidopsis* proteome, including 85 AGPs, 59 EXTs, and 18 PRPs (*Allan et al.,2010*). Numerous studies have shown that EXTs are involved in cell wall reinforcement in higher plants and in defence against pathogen attacks (*Castilleux et al., 2018, 2021*; *Otulak-Koziel et al., 2018*). The cell wall of the unicellular green alga *Chlamydomonas reinhardtii* does not contain cellulose ~~or~~ other polysaccharides, ~~but~~ consists only of an insoluble HRGP framework and several chaotrope-soluble, hydroxyproline-containing glycoproteins (*Jürgen et al., 2009*). The wall enveloping the vegetative and gametic cells (V/G wall) has a highly ordered structure including a chaotrope-soluble crystalline layer assembled with well-characterized HRGPs (*Jeffrey and Ursula, 1992*). The chaotrope-soluble cell wall glycoprotein GP1 is the only polypeptide with an even higher proportion of hydroxyproline occurring in vegetative *C. reinhardtii* cells, and is a constituent of the insoluble cell wall components (*Jürgen et al., 2009*).

The remodelling and signal transduction functions of CWPs play an important role in abiotic stresses such as high temperature (*Pinski et al., 2021*), high salt (*Feng et al., 2018*; *Zhao et al., 2018*), and nutrient deficiency (*Wu et al., 2019*; *Ogden et al., 2018*) in plants. A total of 270 CWPs have been identified in *Oryza sativa* (*Calderan-Rodrigues et al., 2019*), however, the biological functions of rice vegetative cell wall proteins (GP1) involved in salt alkalinity resistance and related signal transduction and protease mechanisms have not been reported. In the current study, the *OsGP1* gene was cloned from rice and ~~its~~ functional site of OsGP1 protein was determined by subcellular localization. The genetic phenotypes of tolerance to soda saline-alkali stress in rice overexpressing *OsGP1* and wild-type were compared to clarify the role of OsGP1 under saline alkaline stress. These results support the involvement of OsGP1 in the stress resistance mechanism of rice cell wall under soda saline-alkali stress.

Materials & Methods

Plant material

O. sativa longjing11 (Lj11) seeds were donated by the research group of Qingyun Bu, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences.

Soda Saline–Alkali Soil Eluent

The soda saline-alkali soil eluent (SAE) used for stress treatment was obtained as described by *Wang et al.* (*2018*). The 0-10 cm soil of heavy alkali patch was collected from the Anda field experiment station (124°53'~125°55'E, 46°01'~47°01'N). The soil was dried, passed through a 5 mm×5 mm sieve, and mixed well. Approximately 4 l of water was poured into 2 l of saline-alkali soil. The mixture was stirred well and left for 12 h (stirring once every 4 h). The mixture was filtered using filter paper to remove impurities, and experimental SAE was obtained. The

different ratios of SAE required in the experiments are shown in Table 1.

Gene cloning ~~and vector construction~~

According to the sequence of Nipponbare *OsGP1* gene (XM_015773690), specific primers OsGP1-F1, R2 (Table S1) were designed. The RNA of Lj11 was reverse-transcribed ~~into~~ cDNA ~~as~~ a template, and the target DNA was amplified by Blend-Taq DNA polymerase. The DNA was purified, inserted into the pMD18-T vector, and then transformed into *Escherichia coli* JM109. The pMD18-T-*OsGP1* plasmid was identified by digestion with restriction endonuclease SalI/BamHI and sent for sequencing. The binary plant expression vector pGWB5-*OsGP1* was constructed by Gateway ~~method~~. The open reading frame (ORF) of *OsGP1* without the stop codon was amplified from the ~~correctly sequenced~~ pMD18-T-*OsGP1* plasmid using specific primers OsGP1-F3, R4 (Table S1), ligated it into the entry vector pENTR/D-TOPO using the pENTR/D-TOPO Cloning Kit (Invitrogen), and then moved to pGWB5 using Gateway LR Clonase Ⅱ Enzyme Mix (Invitrogen). The construction strategy created with Biorender (https://www.biorender.com/) is shown in Figure. S1. The recombinant plasmids were transformed into *E. coli* TOP10 strain and positive colonies were selected and confirmed by PCR to obtain pGWB5-*OsGP1* ~~fused with a green fluorescent protein (GFP) report gene~~ at the C-terminus of *OsGP1* driven by 35S promoter. The schematic diagram of the T-DNA insertion site in pGWB5-*OsGP1* is shown in Fig. 1.

Bioinformatics analysis

The nucleotide sequence obtained by sequencing was used as the comparison benchmark, and the ORF of *OsGP1* was analysed based on NCBI (https://www.ncbi.nlm.nih.gov/). The physicochemical properties of the encoded protein were obtained using the ProtParam (https://web.expasy.org/protparam/). The online tool SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa%20_sopma.html) was used to predict the protein's secondary structure. The tertiary structure was predicted using SWISS-MODEL (https://swissmodel.expasy.org/). Conserved domains were analysed using the online website SMART (https://smart.embl.de). Signal peptides were analysed using SignalP-4.1 (https://services.healthtech.dtu.dk/services/SignalP-4.1/). The Locus ID (Os03g0852400) was found according to *OsGP1* (XM_015773690). *OsGP1* is located at the position of 35895654-35896833 (-strand) on chromosome 3. The sequence at 35896832-35898832 was taken as the *OsGP1* promoter, the promoter elements in this sequence were analysed using Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and mapped using Adobe Illustrator 2021. The sequences with high homology to OsGP1 were obtained by Blastp (NCBI) and aligned using Clustal Omega. The phylogenetic tree was constructed in MEGA6.0 by using the neighbour-joining (NJ) method (bootstrap value, 1,000). The subcellular location of OsGP1 was predicted by PSORT(http://psort1.hgc.jp/form.html).

Subcellular localization

To localize the OsGP1 protein, ~~35S::pGWB5-*OsGP1* with a green fluorescent protein (GFP) was constructed.~~ The recombinant plasmids were transformed into onion epidermal cells using a gene gun (Wealtec, GDS-80, Nevada, USA) according to the manufacturer's instructions, and the

blank pGWB5 was transiently transformed as a control. After incubation at 22 ℃ for 16-24 h in the dark (*Zhao et al., 2013*), the onion epidermal cells were mounted on slides, and the GFP signal was observed under a fluorescence microscope (ZEISS, Axio Imager 2, Gottingen, Germany).

Rice transformation and identification

According to the rice transgenic method of *Upadhyaya et al.* (*2000*) and *Toki et al.* (*2006*), Lj11 seeds were dehulled and sterilized, and the callus was induced on the medium added with 2,4-D. The pGWB5-*OsGP1* plasmid was electrotransformed into *Agrobacterium tumefaciens* EHA105 and used to infect the rice calli. The infected rice calli was screened and differentiated on a hygromycin medium to obtain transgenic T0 generation lines, and the integration of *OsGP1* was detected by PCR by using specific primers OsGP1-F1 and vector primers B5-R (Table S1). The T3 generation transgenic rice was obtained by twice germination selection of 50 mg/l hygromycin medium. The relative expression of *OsGP1* in T3 transgenic lines seedlings was detected by quantitative real-time PCR (qRT-PCR) in a fluorescent quantitative PCR instrument (Agilent, Mx3000p, Waldbronn, Germany). The seeds of overexpressed T3 generation lines were collected for subsequent experiments.

Soda saline-alkaline stress tolerance analysis

To detect soda saline-alkali stress tolerance in rice overexpressing OsGP1, seeds of three independent homozygous transgenic lines (T3-#2, #4, and #5) of the T3 generation and the non-transgenic control (Lj11) were surface sterilized and cultivated in Hoagland nutrient solution at 28 ℃ with 16 h light and 8 h dark photoperiod. Three-leaf stage seedlings were used as treatment materials, and the compound salt mixed with sterilized water and SAE in different ratios was used as the stress treatment solution. The roots of each experimental line were soaked with different ratios of SAE, and the tolerance phenotypes and physiological indices were detected after 7 days. The detection method was based on *Chen and Zhang* (*2016*).

Data Statistical Analysis Methods

Statistical analysis was carried out using IBM SPSS 26 for Windows, and one-way analysis of variance (ANOVA) was used for analysis. Statistical significance was defined as $P \leq 0.05$.

Results

Cloning of *OsGP1* gene

The PCR amplification of the OsGP1 gene resulted in a product of approximately 660 (Fig. 2A), then subsequent cloning process successfully inserted the amplified DNA into the pMD18-T vector, to generate the recombinant plasmid pMD18-T-OsGP1. The plasmid DNA was digested with Sal Ⅰ /BamH Ⅰ , and electrophoresis results showed the DNA fragments were consistent with the PCR product (Fig. 2B). The plasmid was then confirmed by sequencing to obtain an identical nucleotide sequence comparison to the XM_015773690 predicted to be *OsGP1* gene (https://www.ncbi.nlm.nih.gov/pmc/).

Bioinformatics analysis of OsGP1

The ORF of *OsGP1* contains 660 bp nucleotides and encodes 219 amino acids (Fig. 3A). The secondary structure of OsGP1 protein was predicted using SOPMA (Fig. 3B) and the tertiary

structure was predicted by SWISS-MODEL (Fig. 3C), the results showed that it has α-helix and β-fold, accounting for 36.07% and 5.48%, respectively, while the remaining structures are mainly irregularly coiled with a few extended chains. Domain analysis showed that OsGP1 has three conserved domains and one transmembrane domain, of which a conserved domain at amino acid positions 173 to 199 overlaps with the transmembrane domain at 178 to 197 and is not shown (Fig. 3D). According to the signal peptide prediction results of SignalP (Fig. 3E), OsGP1 has a site at the 26th amino acid position that can be recognized and digested by signal peptidase. Hence, a possible signal peptide structure was predicted between the 1st and 25th amino acid sites. The protein interactions of OsGP1 were predicted using STRING (Fig. 3F), and the results showed that it was co-expressed with CESA1, TUBB8, and BC1L4 with scores of 0.646, 0.611, and 0.59, respectively. Cellulose synthase gene 1 (CESA1) is required for the crystallization of β-1,4-glucan microfibrils, which is related to the main mechanism of cell wall formation (*Burn et al., 2002*). Mutants of CESA have lower cellulose content, and there is a negative correlation between cellulose content and plant growth under abiotic stress (*Hori et al., 2020*). *OsCESA9*/*OsCESA9*^{D387N} heterozygous plants enhance plant resistance to salt stress by deregulating the toxicity of ROS, scavenging ROS, and indirectly affecting related genes such as *OsCESA4* and *OsCESA7* (*Ye et al., 2021*). TUBB8 has the same protein sequence as the β-tubulin protein OsTUB8 in *japonica*. *OsTUB8* is mainly expressed in anthers and pollen and is an anther-specific microtubule protein that has a unique role in microtubule formation during anther, pollen development and pollen tube growth. Its expression is upregulated by gibberellin (GA3) and may be involved in GA-regulated anther and/or pollen development (*Yoshikawa et al., 2003*). Proteomic analysis of salt-adapted cells (A120) from *Arabidopsis thaliana* callus showed that compared with wild-type cells, the differentially expressed proteins in A120 cells were strongly associated with cell structure-associated clusters, including cytoskeleton and cell wall biogenesis. Genes such as *TUB4*, *TUB7*, and *TUB9* were induced to be expressed in A120 cells, and the overexpression of *Arabidopsis thaliana TUB9* gene in rice increased the tolerance to salt stress (*Chun et al., 2021*). OsBC1L4 is a plant-specific glycosylphosphatidylinositol (GPI)-anchored protein that is a key regulator of directional cell growth and cellulose crystallinity, and its mutation results in reduced secondary cell wall thickness and cellulose content. The analysis of the *OsGP1* promoter using PlantCARE (Fig. 3G) revealed that it contains the basic promoter elements TATA-box and CAAT-box, the stress-responsive element STRE, the MYB and MYC elements involved in environmental adaptation, the ABRE element involved in abscisic acid (ABA) response, the light response element G-box, LTR involved in low-temperature response, TCA-element involved in salicylic acid (SA) response, HD-Zip 1 involved in differentiation of the palisade mesophyll cells, GCN4 motif involved in endosperm expression, and TGACG-motif involved in methyl jasmonate (MeJA) response. Among them, G-box at position -1953, TCA-element at position -556 and HD-Zip 1 at position -903 had the highest scores of 10, 9 and 8.5, respectively. A total of 43 protein sequences were aligned from Blastp (NCBI) to construct a phylogenetic tree (Fig. 4A). Four proteins in the same branch with OsGP1 were selected for multiple sequence alignment, including that the *O. sativa* Indica group

protein OsI_14363 had 99.54% similarity with the OsGP1 from *O. sativa* japonica group studied here. The similarity of OsGP1 to mucin-1-like from *Oryza brachyantha* was 75.36%, while its similarity to GUJ93 from *Zizania palustris* was 69.61%. The results of Clustal Omega were imported into Jalview, and the analysis showed that most of the conserved regions of the protein were from the 171th amino acid site to the end of the protein (Fig. 4B).

OsGP1 localizes in the cell membrane and cell wall

The subcellular localization of OsGP1 was predicted using PSORT, and the results showed that the probability of OsGP1 protein localization in the cytoplasmic membrane was 46%, 28% in the endoplasmic reticulum membrane, and 10% in the endoplasmic reticulum (Table 2). To further confirm the localization of the OsGP1 protein in vivo, ~~35S::pGWB5-*OsGP1* tagged with GFP~~ was transiently transformed to the onion epidermal cells with a gene gun. Consistent with the predicted cytoplasmic membrane localization, the green fluorescence of the ~~OsGP1-GFP~~ fusion protein was found to be expressed in the cell membrane and cell wall (Fig. 5).

Identification of rice overexpressing *OsGP1*

The rice ~~callus was~~ infected and transformed with *Agrobacterium tumefaciens* EHA105 containing pGWB5-*OsGP1* plasmid and differentiated on the selection medium containing hygromycin. The T0 generation plants were transplanted to pots and genotyped by PCR, and the results showed that *OsGP1* was integrated into the genome of the T0 generation lines (Fig. 6A). Transgenic rice seeds were harvested and screened for two generations under hygromycin to obtain T3 generation seeds. Total RNA was extracted from T3 generation lines and reverse-transcribed into cDNA as a template, and expression of *OsGP1* was detected in the transgenic rice seedlings by qRT-PCR (Fig. 6B). The results showed that the overexpressing lines expressed more than 7-fold of the WT, and the expression of #2 line was up to 11-fold higher than WT. Therefore, the transgenic rice seedlings lines #2, #4, and #5 overexpressing *OsGP1* were selected for subsequent resistance analysis.

Overexpression of OsGP1 enhances soda saline-alkali stress tolerance in rice

Three-leaf stage seedlings of overexpressing *OsGP1* rice lines T3-#2, #4, and #5 were treated with different concentrations of SAE, and the growth phenotypes were observed after 7 days (Fig. 7A). With the increase in SAE content, injuries of rice seedlings gradually increased, the leaves change from green to yellow, and the full leaves shrivelled. The transgenic lines were found to have less damaged leaf than WT. In the treatment groups with H_2O:SAE ratios of 3:1, the WT differed significantly from overexpression lines, with a higher number of dead plants. Fresh weight analysis showed that the overexpression lines have higher fresh weight than WT plants. Similarly, the overexpression lines were significantly taller than that of WT under stress (Fig. 7B, C). Furthermore, the MDA content increased in all lines with increase in SAE concentration, but the overexpression lines had significantly less MDA than WT (Fig. 7D). Also, antioxidant enzyme activity assay showed that overexpression lines had higher SOD and POD enzyme activities than WT. SOD activity was significantly different between WT and overexpression lines under 3:1 and 2:1 concentrations of H_2O:SAE, while POD activity showed the greatest difference when the H_2O:SAE ratio was 4:1 (Fig. 7E, F). These results suggested that

the overexpression of OsGP1 has an effective protective function against plant injury under high pH and high concentrations of ions, and that the overexpression of *OsGP1* improved saline-alkali tolerance in rice.

Discussion

Rice is an important food crop in the world, and its yield is affected by soil salinity and alkalinity. Alkaline salt stress can inhibit the photosynthesis and growth of plants more than neutral salt stress. Under alkaline salt stress conditions, metal ions precipitates, thus decreasing the availability of nutrients in the soil (*Guo et al., 2017*). Under the interaction of high pH and salt ions, alkaline salts are more restrictive than neutral salts in the germination of seeds and the growth of seedlings (*Wang et al., 2022*). When rice plants are subjected to saline-alkali stress, the plant first experiences several changes in the cell wall, including a reduction in cellulose content, disruption of pectin cross-linking, and accumulation of lignin, resulting in the inability of rice to grow normally (*Liu et al., 2022*). CWPs play an important role in plant defense against abiotic stresses. In the current study, OsGP1 was found to be a proline-rich protein containing a signal peptide and a transmembrane structure. The Locus ID of OsGP1 (Os03g0852400) in RAP-DB (https://rapdb.dna.affrc.go.jp/transcript/?name=Os03t0852400-02) labelled as OsAGP31, which was identified in a screen for AGPs in the rice genome. It belongs to the non-classical AGPs which contain an AGP-like region and other atypical regions, and was expressed in roots and panicles (*Ma and Zhao, 2010*). During the sexual reproduction of higher plants, haploid microspores divide through asymmetric mitosis producing a larger vegetative cell (VC) and a smaller generative cell (GC) (*Lu et al., 2021*). The GC divides further into the twin sperm cells (SCs) for double fertilization, whereas the VC exits the cell cycle and serves as a companion cell for the GC and its daughter cell SCs (*Berger and Twell, 2011*). The vegetative cell wall protein OsGP1 is expressed in panicles at the stages of megaspore and microspore development, and STRING interaction analysis showed that it is co-expressed with the tubulin protein TUBB8, indicating that it may play a role in rice reproductive development. The *OsGP1* promoter sequence contains cis-acting elements involved in differentiation of the palisade mesophyll cells (HD-Zip 1), hormone regulation (ABA, SA, and MeJA) and stress response (MYB, MYC and LTR) (*Wang et al., 2019*; *Chen et al., 1995*). HRGPs play an important role in plant biotic and abiotic stress responses, and generally have high expression in roots to enhance the mechanical strength of cell walls to withstand the mechanical force when roots penetrate the soil, high expression of the *HRGPnt3* in tobacco during lateral root formation indicates the same function. (*Keller and Lamb, 1989; Velasquez et al., 2011*). As a member of the HRGP family, the expression of OsGP1 in roots may be related to its function in resisting adversity. The prediction results of the OsGP1-interacting proteins showed that OsGP1 has co-expression with CESA1, TUBB8, BC1L4, and other proteins, these proteins are involved in cellulose synthesis, plant growth and development, hormone signalling regulation, and tubulin synthesis. The promoter analysis, co-expression analysis and organ-specific expression of OsGP1 showed that it may respond to changes in the external environment and internal hormone levels in plants, and play a role in cell wall structure building to maintain cell morphology and protect plants from damage

under stress. Subcellular localization showed that the OsGP1 protein was localized to the cell membrane and cell wall, reflecting the specific expression characteristics of cell wall proteins. Based on protein interaction and localization analysis, OsGP1 may be related to ion channels, cellulose synthesis, or the synthesis and secretion of fructose and other non-cellulosic polysaccharides. Virus infection triggers several inducible basal defense responses, and the HRGP family proteins EXTs are involved in plant cell wall reinforcement and defense. In *potato virus Y* (PVY^{NTN})-infected potatoes, the synthesis of EXTs is induced, whereas the synthesis of the catalytic subunit of cellulose synthase (CesA4) is reduced. The active trafficking of these proteins occurs as a step-in potato cell wall remodelling in response to PVY^{NTN} infection (*Otulak-Kozieł et al., 2018*). Saline-alkali-tolerant rice exhibited higher germination rate, root length, shoot length, fresh weight, and dry weight than sensitive rice under saline-alkali stress. Transgenic rice plants overexpressing *OsGP1* and Lj11 (WT) were treated with different ratios of SAE for saline-alkali stress, the results showed that compared with Lj11, the fresh weight and plant height of transgenic lines were higher, and the degree of leaf yellowing was lower, indicating that the overexpression of *OsGP1* enhances plant resistance to ~~external~~ stresses. The MDA content can reflect the degree of membrane lipid peroxidation, which is an important parameter to reflect the antioxidant capacity of plants (*Gaweł et al., 2004*). SOD and POD are key enzymes for ROS scavenging, and their high intracellular activity usually alleviates the damage of ROS and restores ROS homeostasis in plant cells. The MDA content of overexpressing *OsGP1* lines is lower than that of Lj11, and the SOD and POD activities were significantly higher than those of Lj11 under stress. Thus, the plant cells scavenges the stress-induced ROS by increasing antioxidant enzyme activities to reduce cellular oxidative damage. These results indicate that OsGP1 improves plant stress resistance by increasing the strength of plant cell wall to maintain the homeostasis of the intracellular environment. The current study provides experimental basis for analyzing the structure, function and stress resistance mechanism of rice vegetative cell wall proteins. The elucidation of the molecular function of OsGP1 in cell wall stress resistance and its possible role in plant reproduction and differentiation requires further research.

Conclusion

In the present study, we functionally characterized ~~a new gene, OsGP1,~~ from rice. The results presented here reveal that the OsGP1 plays an essential role in soda salt-alkali stress. Furthermore, we showed that ~~OsGP1~~ overexpressing lines modulate ~~the~~ ROS scavenging, cellular homeostasis, cell wall signaling and formation. Taken together, we conclude that OsGP1 is a unique gene ~~that has~~ the potential to be used as a candidate gene in the molecular breeding of rice to achieve food security and rice tolerance to multiple abiotic stress.

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References

Albenne C, Canut H and Jamet E (2013) Plant cell wall proteomics: The leadership of Arabidopsis

thaliana. *Frontiers in Plant Science* 4, 111.
Allan MS, Brian K, Jens L, Dazhang G and Lonnie RW (2010) A Bioinformatics Approach to the Identification, Classification, and Analysis of Hydroxyproline-Rich Glycoproteins. *Plant Physiology* 153(2), 485–513.
Berger F and Twell D (2011) Germline specification and function in plants. *Annual review of plant biology* 62, 461–484.
Burn JE, Hocart CH, Birch RJ, Cork AC and Williamson RE (2002) Functional analysis of the cellulose synthase genes CesA1, CesA2, and CesA3 in Arabidopsis. *Plant physiology* 129(2), 797–807.
Calderan-Rodrigues MJ, Guimarães Fonseca J, de Moraes FE, Vaz Setem L, Carmanhanis Begossi A and Labate CA (2019) Plant Cell Wall Proteomics: A Focus on Monocot Species, Brachypodium distachyon, Saccharum spp. and Oryza sativa. *International journal of molecular sciences* 20(8), 1975.
Castilleux R, Plancot B, Ropitaux M, Carreras A, Leprince J, Boulogne I, Follet-Gueye ML, Popper ZA, Driouich A and Vicré M (2018) Cell wall extensins in root-microbe interactions and root secretions. *Journal of experimental botany* 69(18), 4235–4247.
Castilleux R, Plancot B, Vicré M, Nguema-Ona E and Driouich A (2021) Extensin, an underestimated key component of cell wall defence? *Annals of botany* 127(6), 709–713.
Chen T, Zhang B (2016) Measurements of Proline and Malondialdehyde Content and Antioxidant Enzyme Activities in Leaves of Drought Stressed Cotton. *BIO-PROTOCOL* 6(17).
Chen Z, Malamy J, Henning J, Conrath U, Sánchez-Casas P, Silva H, Ricigliano J and Klessig DK (1995) Induction, modification, and transduction of the salicylic acid signal in plant defense responses. *Proceedings of the National Academy of Sciences of the United States of America* 92(10), 4134–4137.
Chun HJ, Baek D, Jin BJ, Cho HM, Park MS, Lee SH, Lim LH, Cha YJ, Bae DW, Kim ST, Yun DJ and Kim MC (2021) Microtubule Dynamics Plays a Vital Role in Plant Adaptation and Tolerance to Salt Stress. *International journal of molecular sciences* 22(11), 5957
Feng W, Kita D, Peaucelle A, Cartwright HN, Doan V, Duan Q, Liu MC, Maman J, Steinhorst L, Schmitz-Thom I, Yvon R, Kudla J, Wu HM, Cheung AY and Dinneny JR (2018) The FERONIA Receptor Kinase Maintains Cell-Wall Integrity during Salt Stress through Ca^{2+} Signaling. *Current biology* 28(5), 666–675.
Gaweł S, Wardas M, Niedworok E and Wardas P (2004) Malondialdehyde (MDA) as a lipid peroxidation marker. *Wiadomosci lekarskie 57*(9-10), 453–455.
Guo R, Shi L, Yan C, Zhong X, Gu F, Liu Q, Xia X and Li H (2017) Ionomic and metabolic responses to neutral salt or alkaline salt stresses in maize (Zea mays L.) seedlings. *BMC plant biology* 17(1), 41.
Herger A, Dünser K, Kleine-Vehn J and Ringli C (2019) Leucine-Rich Repeat Extensin Proteins and Their Role in Cell Wall Sensing. *Current biology* 29(17), R851-R858.

Hori C, Yu X, Mortimer JC, Sano R, Matsumoto T, Kikuchi J, Demura T and Ohtani M (2020) Impact of abiotic stress on the regulation of cell wall biosynthesis in *Populus trichocarpa*. *Plant Biotechnology* 37(3).

Hunt L, Amsbury S, Baillie A, Movahedi M, Mitchell A, Afsharinafar M, Swarup K, Denyer T, Hobbs JK, Swarup R, Fleming AJ and Gray JE (2017) Formation of the Stomatal Outer Cuticular Ledge Requires a Guard Cell Wall Proline-Rich Protein. *Plant Physiol* 174(2), 689-699.

Jamet E, Albenne C, Boudart G, Irshad M, Canut H and Pont-Lezica R (2008) Recent advances in plant cell wall proteomics. *Proteomics* 8(4), 893–908.

Jamet E and Dunand C (2020) Plant Cell Wall Proteins and Development. *International journal of molecular sciences* 21(8), 2731.

Jeffrey PW and Ursula WG (1992) Zygote and vegetative cell wall proteins in *Chlamydomonas reinhardtii* share a common epitope, $(SerPro)_X$. *Plant Science* 83(1), 65-76.

Jiang L, Zhang L, Zhao H and Gong L (2019) Physical suitability evaluation and potential development of reserve cultivated land resources in Songnen Plain. *Chinese Journal of Agricultural Resources and Regional Planning* 10, 15-25.

Jürgen V, Ronald F and Johannes W (2009) The chaotrope-soluble glycoprotein GP1 is a constituent of the insoluble glycoprotein framework of the *Chlamydomonas* cell wall. *FEMS Microbiology Letters* 291(2), 209-215.

Keller B and Lamb CJ (1989) Specific expression of a novel cell wall hydroxyproline-rich glycoprotein gene in lateral root initiation. *Genes & development* 3(10), 1639–1646.

Li Q, Li X, Li X, Wang Z, Song C and Zhang G (2003) Sodium bicarbonate soil management and utilization in Songnen Plain. *Resources Science* 01, 15-20.

Liang W, Ma X, Wan P and Liu L (2018) Plant salt-tolerance mechanism: A review. *Biochemical and biophysical research communications* 495(1), 286–291.

Liu J, Zhang W, Long S and Zhao C (2021) Maintenance of Cell Wall Integrity under High Salinity. *International journal of molecular sciences* 22(6), 3260.

Lu Y, Song Y, Liu L and Wang T (2021) DNA methylation dynamics of sperm cell lineage development in tomato. *The Plant journal : for cell and molecular biology* 105(3), 565–579.

Ma H and Zhao J (2010) Genome-wide identification, classification, and expression analysis of the arabinogalactan protein gene family in rice (*Oryza sativa L.*). *Journal of Experimental Botany* 61(10), 2647-2668.

Ogden M, Hoefgen R, Roessner U, Persson S and Khan GA (2018) Feeding the Walls: How Does Nutrient Availability Regulate Cell Wall Composition? *International journal of molecular sciences* 19(9), 2691.

Otulak-Kozieł K, Kozieł E and Lockhart BEL (2018) Plant Cell Wall Dynamics in Compatible and Incompatible Potato Response to Infection Caused by Potato Virus Y (PVY^{NTN}). *International journal of molecular sciences* 19(3), 862.

Pastori GM and Foyer CH (2002) Common components, networks, and pathways of cross-tolerance to stress. The central role of "redox" and abscisic acid-mediated controls. *Plant physiology* 129(2), 460-468.

Pinski A, Betekhtin A, Skupien-Rabian B, Jankowska U, Jamet E and Hasterok R (2021) Changes in the Cell Wall Proteome of Leaves in Response to High Temperature Stress in Brachypodium distachyon. *International journal of molecular sciences* 22(13), 6750.

Showalter AM, Keppler B, Lichtenberg J, Gu D and Welch LR (2010) A bioinformatics approach to the identification, classification, and analysis of hydroxyproline-rich glycoproteins. *Plant physiology* 153(2), 485–513.

Velasquez SM, Ricardi MM, Dorosz JG, Fernandez PV, Nadra AD, Pol-Fachin L, Egelund J, Gille S, Harholt J, Ciancia M, Verli H, Pauly M, Bacic A, Olsen CE, Ulvskov P, Petersen BL, Somerville C, Iusem ND and Estevez JM (2011) O-glycosylated cell wall proteins are essential in root hair growth. *Science* 332(6036), 1401–1403.

Toki S, Hara N, Ono K, Onodera H, Tagiri A, Oka S and Tanaka H (2006) Early infection of scutellum tissue with Agrobacterium allows high-speed transformation of rice. *The Plant journal: for cell and molecular biology* 47(6), 969–976.

Upadhyaya NM, Surin B, Ramm K, Gaudron J, Schünmann PHD Taylor W, Waterhouse PM and Wang MB (2000) Agrobacterium-mediated transformation of Australian rice cultivars Jarrah and Amaroo using modified promoters and selectable markers. *Australian Journal of Plant Physiology* 27(3), 201-210.

Vaidyanathan H, Sivakumar P, Chakrabarty R and Thomas G (2003) Scavenging of reactive oxygen species in NaCl-stressed rice (oryza sativa L.) — differential response in salt-tolerant and sensitive varieties. *Plant Science* 165(6), 1411-1418.

Wang H, Takano T and Liu S (2018) Screening and Evaluation of Saline–Alkaline Tolerant Germplasm of Rice (Oryza sativa L.) in Soda Saline–Alkali Soil. *Agronomy* 8(10), 205-205.

Wang L, Chen J and Li J (2019) Cloning and Expression Analysis of AtNHX6 Gene Promoter from the Arabidopsis thaliana. *Acta Botanica Boreali-Occidentalia Sinica* 39(02), 191-198.

Wang J, Lin C, Han Z, Fu C, Huang D and Cheng H (2022) Dissolved nitrogen in salt-affected soils reclaimed by planting rice: How is it influenced by soil physicochemical properties? *Science of The Total Environment* 824,153863.

Wang W, Zhang F, Sun L, Yang L, Yang Y, Wang Y, Siddique KHM and Pang J (2022) Alkaline Salt Inhibits Seed Germination and Seedling Growth of Canola More Than Neutral Salt. *Frontiers in plant science* 13, 814755.

Wang Y, Zhang R, Liu Y, Li R, Ge J, Deng S, Zhang X, Chen Y, Wei H and Dai Q (2022) Rice response to salt stress and research progress in salt tolerance mechanism. *Chinese Journal of Rice Science* 02, 105-117.

Wu W, Zhu S, Chen Q, Lin Y, Tian J and Liang C (2019) Cell Wall Proteins Play Critical Roles in Plant Adaptation to Phosphorus Deficiency. *International journal of molecular sciences* 20(21), 5259.

Ye Y, Wang S, Wu K, Ren Y, Jiang H, Chen J, Tao L, Fu X, Liu B and Wu Y (2021) A Semi-

Dominant Mutation in OsCESA9 Improves Salt Tolerance and Favors Field Straw Decay Traits by Altering Cell Wall Properties in Rice. *Rice* 14(1), 19.

Yoshikawa M, Yang G, Kawaguchi K and Komatsu S (2003) Expression analyses of beta-tubulin isotype genes in rice. *Plant & cell physiology* 44(11), 1202–1207.

Zhang N, Li Y, Zhu L and He G (2003) Review of the Research on the Classification of the Genus Oryza. *Chinese Journal of Rice Science* 04, 104-108

Zhao C, Zayed O, Yu Z, Jiang W, Zhu P, Hsu CC, Zhang L, Tao WA, Lozano-Durán R and Zhu JK (2018) Leucine-rich repeat extensin proteins regulate plant salt tolerance in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* 115(51),13123-13128.

Zhao JF, Gao YJ, Liu YL and Cao Y (2013) Recombinant plasmid transform into the cuticle of onion by gene gun method. *Journal of Hengshui University* 15(01), 31-34.

Figure 1

Schematic diagram of the T-DNA ~~insertion site~~ in ~~pGWB5 *OsGP1*~~ plasmid

Figure 2

Electropherogram of *OsGP1* DNA

A, Electropherogram of PCR amplification product from rice cDNA. M, Marker, DL2000; #1 and #2, PCR amplification products of *OsGP1*. B, Recombinant pMD18-T-*OsGP1* plasmid digested with SalI/BamHI. M: Marker, DL5000; #1, pMD18-T-*OsGP1* plasmid; #2, pMD18-T-*OsGP1* plasmids digested with SalI/BamHI.

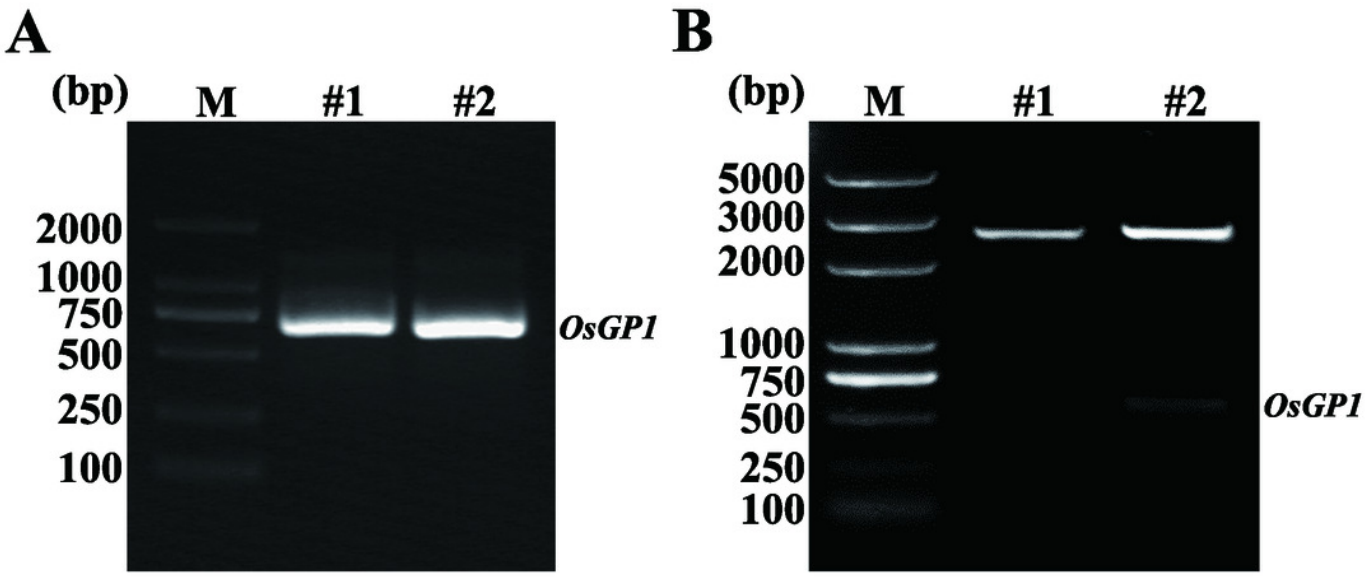
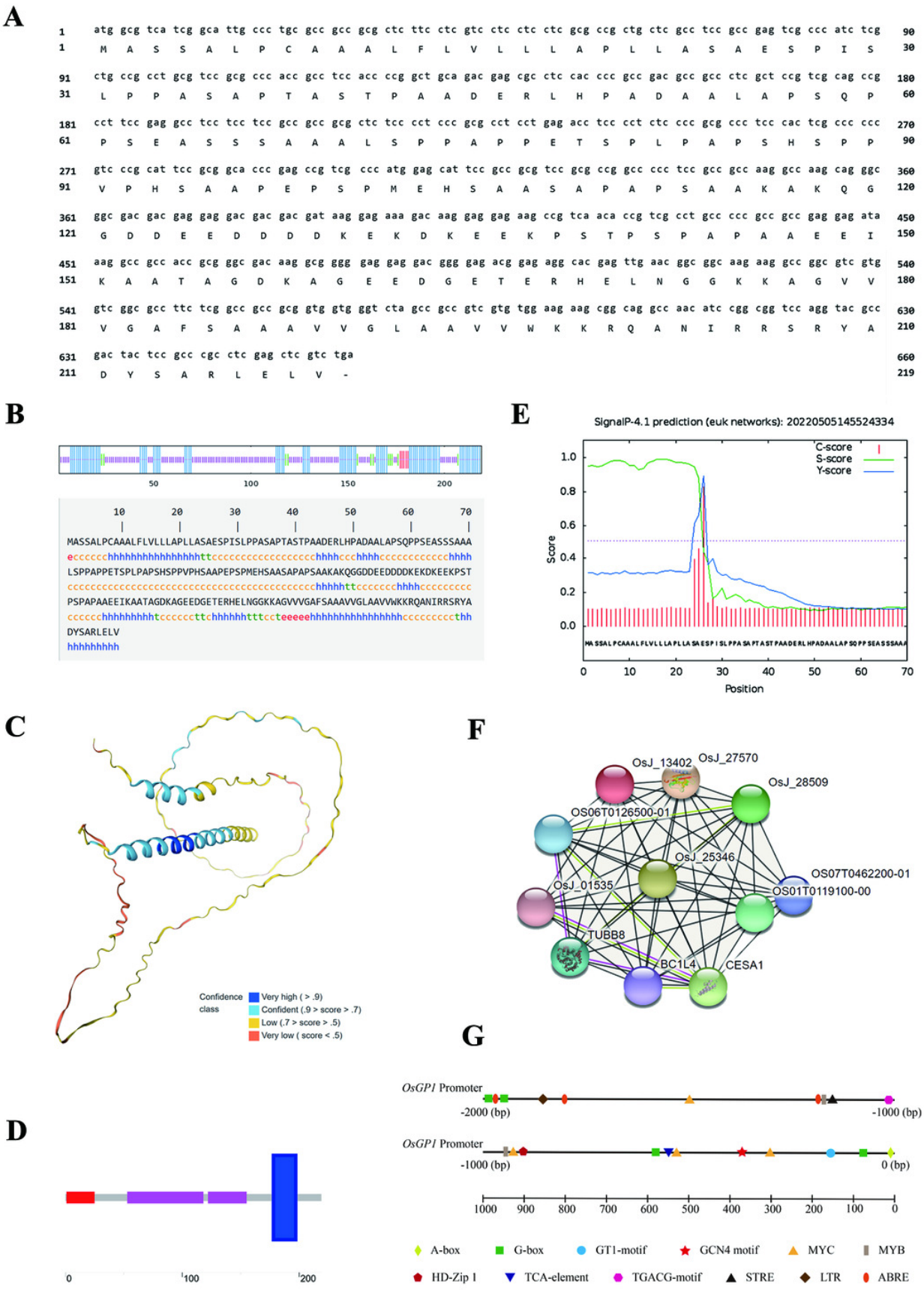


Figure 3

The bioinformatics analysis of OsGP1

A, Nucleotide sequence and deduced amino acid sequence of *OsGP1* coding region. B, Secondary structure prediction. Blue, α-helix (h); green, β-turn (t); red, extended strand (e); purple, random coil (c). C, Tertiary structure prediction. The model was constructed with Q851X5_ORYSJ as the template. The GMQE (Global Model Quality Estimate) score is 0.62 and the colours represent different model confidence scores. D, Prediction of conserved domains of OsGP1 protein. Red, signal peptide; blue, transmembrane region; purple, low complexity region. E, Prediction of the signal peptide. The S-score is higher in the signal peptide region, the C-score is highest at the cleavage site, the Y-score is a parameter that comprehensively considers the S-score and C-score, and the Y-max score is the putative cleavage site. F, Protein interaction relationship. Different coloured lines represent different interaction types. Black, co-expression; purple, experimentally determined; yellow, textmining. G, Analysis of *OsGP1* promoter. All cis-acting elements selected for display have a PlantCARE matrix score ≥ 5.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1 atg gcg tca tcg gca ttg ccc tgc gcc gcc gcg ctc ttc ctc gtc ctc ctc ctc gcg ccg ctg ctc gcc tcc gcc gag tcg ccc atc tcg 90
1 M A S S A L P C A A A L F L V L L L A P L L A S A E S P I S 30
91 ctg ccg cct gcg tcc gcg ccc acc gcc tcc acc ccg gct gca gac gag cgc ctc cac ccc gcc gac gcc gcc ctc gct ccg tcg cag ccg 180
31 L P P A S A P T A S T P A A D E R L H P A D A A L A P S Q P 60
181 cct tcc gag gcc tcc tcc tcc gcc gcc gcg ctc tcc cct ccc gcg cct cct gag acc tcc cct ctc ccc gcg ccc tcc cac tcg ccc ccc 270
61 P S E A S S S A A A L S P P A P P E T S P L P A P S H S P P 90
271 gtc ccg cat tcc gcg gca ccc gag ccg tcg ccc atg gag cat tcc gcc gcg tcc gcg ccg gcc ccc tcc gcc gcc aag gcc aag cag ggc 360
91 V P H S A A P E P S P M E H S A A S A P A P S A A K A K Q G 120
361 ggc gac gac gag gag gac gac gac gat aag gag aaa gac aag gag gag aag ccg tca aca ccg tcg cct gcc ccc gcc gcc gag gag ata 450
121 G D D E E D D D D K E K D K E E K P S T P S P A P A A E E I 150
451 aag gcc gcc acc gcg ggc gac aag gcg ggg gag gag gac ggg gag acg gag agg cac gag ttg aac ggc ggc aag aag gcc ggc gtc gtg 540
151 K A A T A G D K A G E E D G E T E R H E L N G G K K A G V V 180
541 gtc ggc gcc ttc tcg gcc gcc gcg gtg gtg ggt cta gcc gcc gtc gtg tgg aag aag cgg cag gcc aac atc cgg cgg tcc agg tac gcc 630
181 V G A F S A A A V V G L A A V V W K K R Q A N I R R S R Y A 210
631 gac tac tcc gcc cgc ctc gag ctc gtc tga 660
211 D Y S A R L E L V - 219
B
50 100 150 200
10 20 30 40 50 60 70
MASSALPCAAALFLVLLLAPLLASAESPISLPPASAPTASTPAADERLHPADAALAPSQPPSEASSSAAA
LSPPAPPETSPLPAPSHSPPVPHSAAPEPSPMEHSAASAPAPSAAKAKQGGDDEEDDDKEKDKEEKPST
PSPAPAAEEIKAATAGDKAGEEDGETERHELNGGKKAGVVVGAFSAAAVVGLAAVVWKKRQANIRRSRYA
DYSARLELV
E
SignalP-4.1 prediction (euk networks): 20220505145524334
C-score
S-score
Y-score
Score
Position
C
Confidence class
Very high (> .9)
Confident (.9 > score > .7)
Low (.7 > score > .5)
Very low (score < .5)
F
OsJ_13402
OsJ_27570
OsJ_28509
OS06T0126500-01
OsJ_25346
OS07T0462200-01
OsJ_01535
OS01T0119100-00
TUBB8
BC1L4
CESA1
D
0 100 200
G
OsGP1 Promoter
-2000 (bp)
-1000 (bp)
OsGP1 Promoter
-1000 (bp)
0 (bp)
1000 900 800 700 600 500 400 300 200 100 0
A-box
G-box
GT1-motif
GCN4 motif
MYC
MYB
HD-Zip 1
TCA-element
TGACG-motif
STRE
LTR
ABRE

Figure 4

Phylogenetic tree and multiple sequence alignment of OsGP1 protein

A, Phylogenetic tree. B, Multiple sequence alignment ~~of four~~ sequences in the same branch with OsGP1.

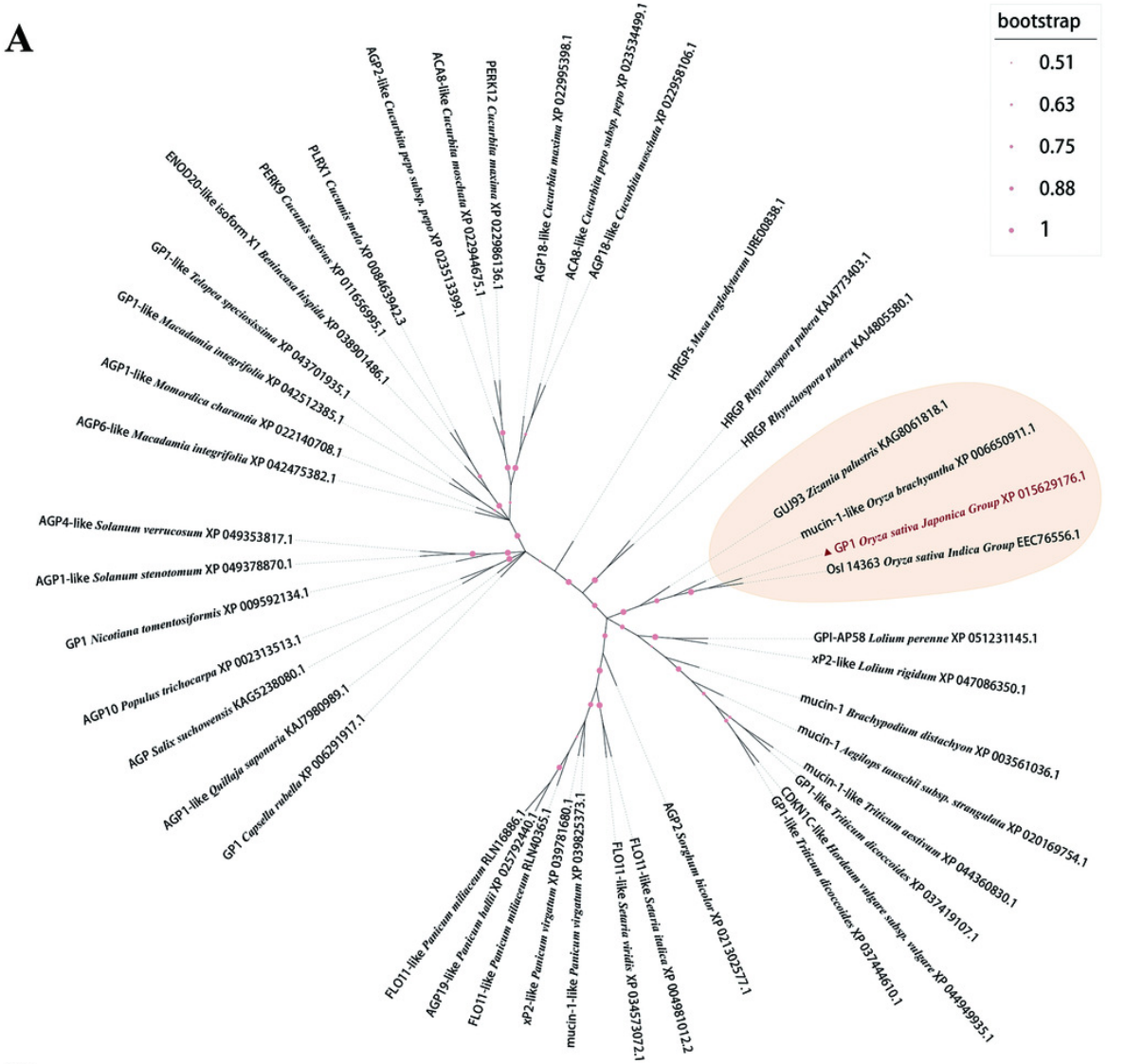
A
bootstrap
0.51
0.63
0.75
0.88
1
AGP2-like Cucurbita pepo subsp. pepo XP 023513399.1
ACA8-like Cucurbita moschata XP 022944675.1
PERK12 Cucurbita maxima XP 022986136.1
AGP18-like Cucurbita maxima XP 022995398.1
ACA8-like Cucurbita pepo subsp. pepo XP 023534499.1
AGP18-like Cucurbita moschata XP 022958106.1
PLRK1 Cucumis melo XP 008463942.3
PERK9 Cucumis sativus XP 011656995.1
ENOD20-like isoform X1 Benincasa hispida XP 038901486.1
GP1-like Telopea speciosissima XP 043701935.1
GP1-like Macadamia integrifolia XP 042512385.1
AGP1-like Momordica charantia XP 022140708.1
AGP6-like Macadamia integrifolia XP 042475382.1
AGP4-like Solanum verrucosum XP 049353817.1
AGP1-like Solanum stenotomum XP 049378870.1
GP1 Nicotiana tomentosiformis XP 009592134.1
AGP10 Populus trichocarpa XP 002313513.1
AGP Salix suchowensis KAG5238080.1
AGP1-like Quillaja saponaria KAJ7980989.1
GP1 Capsella rubella XP 006291917.1
FLO11-like Panicum miliaceum RLN16886.1
AGP19-like Panicum hallii XP 025792440.1
FLO11-like Panicum miliaceum RLN40365.1
xP2-like Panicum virgatum XP 039781680.1
mucin-1-like Panicum virgatum XP 039825373.1
FLO11-like Setaria viridis XP 034573072.1
FLO11-like Setaria italica XP 004981012.2
AGP2 Sorghum bicolor XP 021302577.1
GP1-like Triticum dicoccoides XP 037446610.1
CDKN1C-like Hordeum vulgare subsp. vulgare XP 044949935.1
GP1-like Triticum dicoccoides XP 037419107.1
mucin-1-like Triticum aestivum XP 044360830.1
mucin-1 Aegilops tauschii subsp. strangulata XP 020169754.1
mucin-1 Brachypodium distachyon XP 003561036.1
xP2-like Lolium rigidum XP 047086350.1
GPI-AP58 Lolium perenne XP 051231145.1
HRGP Rhynchospora pubera KAJ4805580.1
HRGP Rhynchospora pubera KAJ4773403.1
HRGP3 Musa troglodytarum URE00838.1
GUJ93 Zizania palustris KAG8061818.1
mucin-1-like Oryza brachyantha XP 006650911.1
GP1 Oryza sativa Japonica Group XP 015629176.1
Osl 14363 Oryza sativa Indica Group EEC76556.1
B
GP1
Osl_14363
mucin-1-like
GUJ93
Consensus

Figure 5

Subcellular localization of ~~OsGP1-GFP~~ fusion protein in onion epidermal cells

GFP and OsGP1-GFP driven by 35S promoter under fluorescence, bright field, and merged views. Bar, 50 μm.

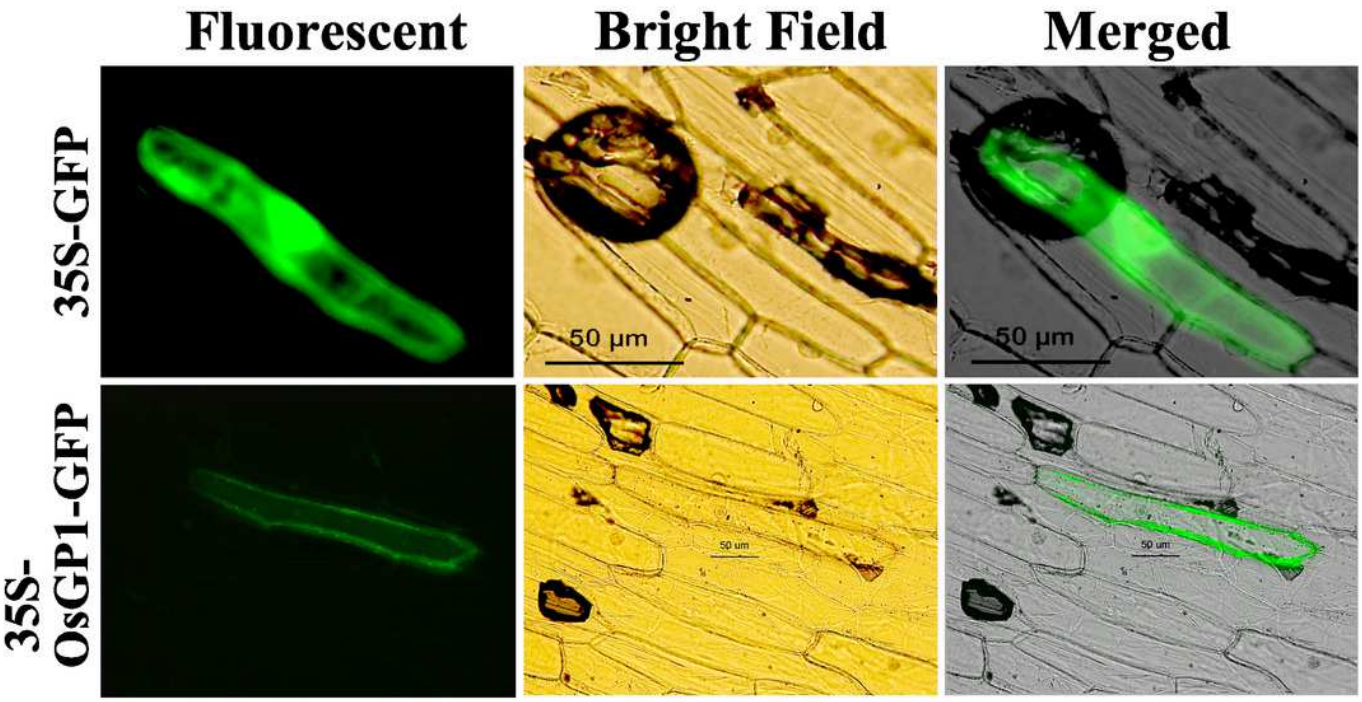

Figure 6

Identification of *OsGP1* transgenic rice lines

A, Identification of T0 generation transgenic rice. M, Marker, DL2000. B, Expression ~~detection~~ of *OsGP1* in T3 generation transgenic rice. WT, Wild-type line, Lj11; #1-#6, *OsGP1* transgenic lines. Asterisks indicate significant mean differences between *OsGP1* overexpression lines and Lj11 (**$P \leq 0.01$). Data represent the mean ± SD of three replicates.

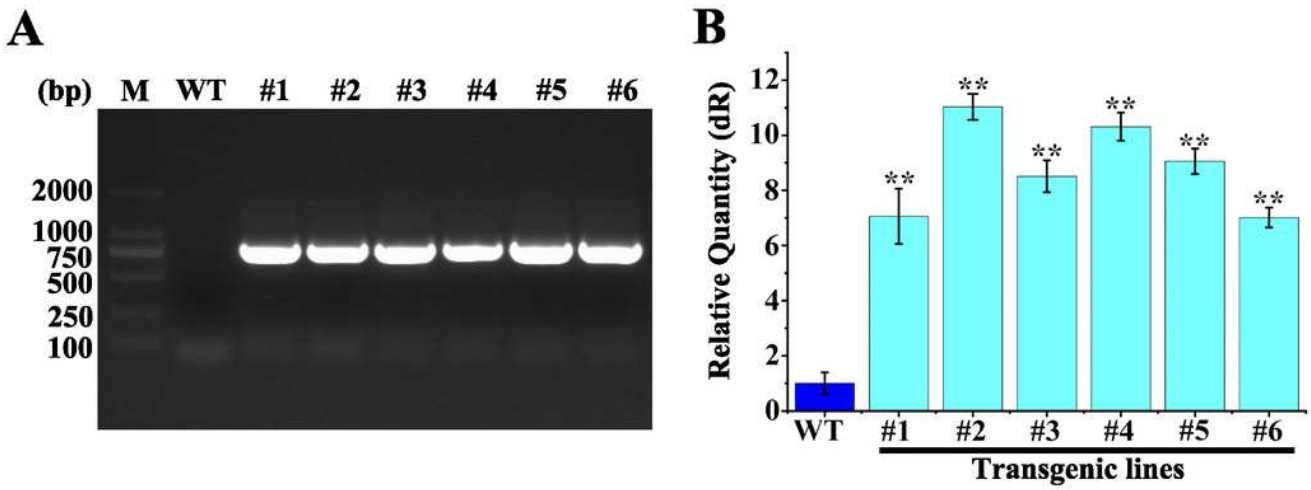

Figure 7

Tolerance analysis of rice overexpressing *OsGP1* to soda saline-alkali stress

The three-leaf stage overexpression lines T3-#2, #4, #5 and Lj11 seedlings were treated with different ratios of SAE for 7 days, and water as control. A, Growth phenotypes. B, Fresh weight of 5 seedlings. C, Plant height. D, MDA content. E, SOD activity. F, POD activity. Data show the mean ± SEM of three replicates. Statistical differences are labelled with different letters using Tukey test ($P \leq 0.05$, one-way ANOVA).

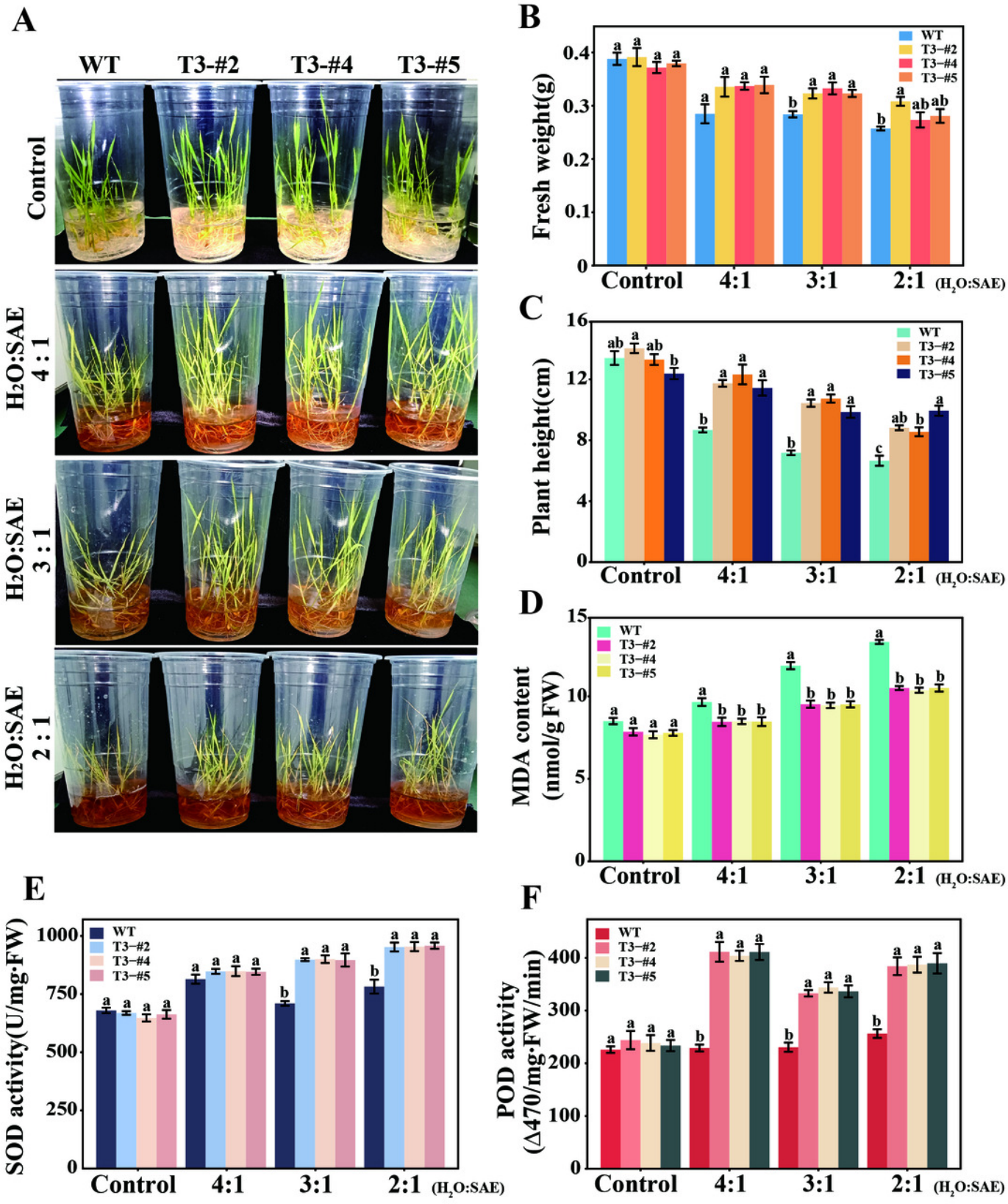
A
WT
T3-#2
T3-#4
T3-#5
Control
H2O:SAE 4 : 1
H2O:SAE 3 : 1
H2O:SAE 2 : 1
B
Fresh weight(g)
C
Plant height(cm)
D
MDA content (nmol/g FW)
E
SOD activity(U/mg·FW)
F
POD activity (Δ470/mg·FW/min)
Control
4:1
3:1
2:1 (H2O:SAE)

Table 1(on next page)

Configuration and characteristics of different ratios of soda saline-alkali soil eluent

Independent triplicate measurements were averaged and the standard deviation (SD) was calculated.

Leachate	Configuration method	EC (μS·cm-1)	pH
SAE (Stock solution)	100ml SAE	10290±36	9.65±0.08
H_2O:SAE=2:1	66ml H_2O + 33ml SAE	3903±38	9.21±0.06
H_2O:SAE=3:1	75ml H_2O + 25ml SAE	2927±35	9.08±0.05
H_2O:SAE=4:1	80ml H_2O + 20ml SAE	2530±26	8.95±0.06
Control (H_2O)	100ml H_2O	82.6±0.1	6.98±0.04

1

Table 2(on next page)

Subcellular localization prediction of OsGP1

The subcellular location of OsGP1 was predicted by PSORT (http://psort1.hgc.jp/form.html).

location	probability
plasma membrane	0.460
endoplasmic reticulum membrane	0.280
endoplasmic reticulum	0.100
outside	0.100

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