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Full Length Article

Multiple Stress Responsive WRKY Transcription Factor, *StWRKY2*, Enhances Drought and Late Blight Resistance in Transgenic Potato

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Abstract

Potato is one of the most important food crops both in terms of human consumption and is surrounded with wide range of environmental challenges including abiotic and biotic stresses. Drought and late blight are two major stress factors that seriously affect potato production around the world. The WRKY transcription factors are key players to fine tune plant responses under various stresses. In current study, an attempt to characterize the gene *Solanum tuberosum* WRKY2 (*StWRKY2*) has been described. The results show that *StWRKY2* is involved in resistance mechanism to abiotic and biotic stress. *StWRKY2* was localized in the nucleus and showed transactivation activity in yeast cells. *StWRKY2* was induced by various stress conditions and hormones in *E. coli* as well as in potato plants. Potato plants overexpressing *StWRKY2* enhanced its tolerance to drought stress via different mechanisms such as preventing water loss by inducing stomatal closure and increased ROS-scavenging ability under drought stress conditions. Moreover, it enhanced the potato resistance to late blight disease through the induction of pathogenesis-related (PR) genes expression. Overall, our findings manifested that *StWRKY2* is a potential candidate for biotic and abiotic resistance and could be used for engineering drought tolerance and late blight resistance in other plants specifically solanaceous crops. © 2020 Friends Science Publishers

Keywords: Drought; *Phytophthora infestans*; *StWRKY2*; *PR* genes; Stomatal closure; ROS scavenging

Introduction

Potato (*Solanum tuberosum* L.) is an important crop all over the world, because it offers relatively easier cultivation practices and provides a rich source of valuable nutrients for the human diet (Beals 2018). Like all plants growing in natural habitats, potato is exposed to various environmental stresses including abiotic and biotic factors (Dahal *et al*. 2019). Drought is a vital factor that negatively affects potato growth and development (Fahad *et al*. 2017). Intense drought causes a radical decline in crop yield through negative influences on plant growth, physiology, protection and reproduction (Barnabas *et al*. 2008). Poor germination, impaired seedling establishment, and bad growth are initial effects of drought on plants (Farooq *et al*. 2009; Shekari *et al*. 2015; Iqbal *et al*. 2017; Per *et al*. 2017; Hussain *et al*. 2018). Vegetative growth parameters including number of leaves/branch, total leaf area, height of plant, fresh and dry weight are also severely reduced under the water limiting conditions (Singh *et al*. 2015; Fahad *et al*. 2017; Alzahrani and Rady 2019; Sattar *et al*. 2019a). Potato is generally considered as drought-sensitive crop (Aliche *et al*. 2018) and if water requirements are not met properly, then huge yield losses of up to 79% can be expected (Prasad *et al*. 2015), which pose a great threat to its global production.

Potato late blight is a devastating potato disease which is caused by the oomycete pathogen *Phytophthora infestans*. Due to its severe damage to the crop, huge economic losses as much as $6.7 billion have been reported which are increasing annually (Lal *et al*. 2018). Molecular mechanism

of the durable resistance to pathogen was previously reported through different efforts to understand the relation between the pathogen and related pathogenesis (*PR*) proteins (Shi *et al*. 2012). Among these proteins, *PR2*, *PR3*, *PR9* and *PR10* genes were previously isolated from *Arabidopsis thaliana* and *S. tuberosum* leaves challenged by *P. infestans* and reported as key genes in the pathogen's resistance pathway (Oide *et al*. 2013; Yang *et al*. 2018). To address this global challenge, many strategies have been established in order to understand molecular mechanisms of broad-spectrum disease resistance. Among these efforts, using available transformation techniques to introduce genes of interest that provide higher agronomic performance both under biotic and abiotic stress in transgenic potato is increasing that leads to the development of modern biotech crops (Halterman *et al*. 2016).

In plants, the WRKY transcription factors (TFs) are ubiquitously distributed across various species including lower plants, however with few exceptions and constitute one of the largest transcription factor families. According to earlier classification considering their structural characteristics, WRKY proteins can be divided into three main groups: WRKY members that contain two WRKY domains are put together in group I, while WRKY group II and III contains only one WRKY domain (Chen and Liu 2019). WRKY TFs act as auto and cross regulators and thus participate in different plant processes at multiple levels including their role in modulating other plant TFs (Yan *et al*. 2013). An important feature of a WRKY protein is the presence of a DNA binding domain usually at C-terminus which contains a conserved WRKYGQK sequence and a zinc motif [CX4–5CX22–23HXH] (Ma *et al*. 2017).

Previously, important role of WRKY proteins have been implicated in various abiotic and biotic stresses as well as in phytohormone-mediated signal transduction in plants (Shahzad *et al*. 2016; Ma *et al*. 2017). In general, WRKY TFs are important for initiating rather a sophisticated network of signaling to regulate specialized metabolism during stress situations in plants (Schluttenhofer and Yuan 2015). Several WRKY genes were reported in various crop plants to confer multiple stress resistance. For instance, two cotton WRKY genes (*GhWRKY39-1* and *GhWRKY40*) were found to regulate resistance against *R. solanacearum* and salt stress in transgenic tobacco plants (Shi *et al*. 2014). Another study showed that *TaWRKY44*, a WRKY TF from wheat, regulate positive response simultaneously for drought, salt and osmotic stress by eliminating ROS thereby activation of cellular antioxidant system or indirectly by upregulation of downstream stress-responsive genes (Wang *et al*. 2015). In *brachypodium distachyon*, upregulation of 15 *BdWRKY* genes were recorded which play important role for initiating defense mechanisms against *Fusarium graminearum* and *Magnaporthe grisea* (Wen *et al*. 2014). Moreover, WRKY TFs play crucial role in salicylic acid (SA) and abscisic acid (ABA) mediated signaling pathways mainly through controlling the expression of stress-inducible genes (Jiang *et al*. 2014; Fan *et al*. 2016). Noticeably, there has been a little relevant research on the WRKY family in one of the world's most important crop potato, however, only *StWRKY1* has been functionally characterized (Yogendra *et al*. 2015; Shahzad *et al*. 2016).

In the current study, we have characterized another WRKY from potato, *StWRKY2* and achieved tempting results including enhanced tolerance to drought and strong resistance to *P. infestans* along with its response to various phytohormones in transgenic potato lines. Further, the underlying resistance mechanism of *StWRKY2* has also been elucidated. Using *StWRKY2* as potential candidate gene, this study will further pave the way for functional breeding programs aimed at producing stress-resistant crop plants.

Materials and Methods

Plant material, growth conditions, vector construction and transformation

The cultivated potato (cv. E-potato 3, E3) was used in this study as control (untransformed E3) and to generate the transgenic plants. Potato plants were normally grown under greenhouse conditions: 25°C ± 2°C, 14/10 h light and dark cycle, respectively. *StWRKY2* was amplified from the cDNA and genomic DNA of *S. tuberosum*, using specific primers for *StWRKY2* (forward and reverse). After successful amplification, the PCR product was cloned into the pMD18-T vector and samples were later sequenced to selective the positive clone. For overexpression vector construct, we simultaneously digested the *StWRKY2* plasmid (in pMD18-T vector) and plant binary vector pBI121 with *XbaI* and *KpnI* followed by ligation with T4 DNA ligase. To construct GFP reporter construct for subcellular localization, we used pCMV-GFP vector. The *StWRKY2* cDNA was fused in frame with GFP (35S::*StWRKY2*::GFP) and selected plasmid was later injected into tobacco (*N. tabacum*) leaves. The leaves were later observed for GFP signals using a confocal microscope (Zeiss, LSM510, Germany).

Bioinformatics analysis

For homology search, we used the full length amino acid sequence of *S. tuberosum* WRKY2, *StWRKY2*, obtained through utilizing the online public database NCBI (http://www.ncbi.nlm.nih.gov). For multiple sequence alignments, we computed the amino acid sequences of *StWRKY2* with its homologues using Clustal W program employing standard parameters. In order to analyze evolutionary relationship of *StWRKY2* with its homologues in selected plant species, we generated phylogenetic tree by neighbor-joining method utilizing MEGA 7.0. To find out cis-elements in the promoter region, firstly we obtained 1.5 Kb upstream sequences from potato genome browser and analyzed it for putative cis-elements using the PlantCARE and PLACE databases.

Plant regeneration

The plasmid (PBI121-*StWRKY2*) was first introduced into *Agrobacterium tumefaciens* strain C58 and subsequently cultured in liquid LB broth containing 50 mg L^{-1} kanamycin and 50 mg L^{-1} rifampicin. The culture was then incubated at 28°C and kept on continuous shaker until the optical density reached at 0.5 $O.D_{600}$. For plant regeneration, we used microtubers that were obtained from the E3 plantlets. These microtubers were later dipped in 20 mL bacterial solution for 5–10 min in petri dishes as described by previous study (Huai-Jun *et al*. 2003). To obtain efficient number of healthy plantlets, we prepared 2-mm discs from microtubers and transfer them onto petri dishes. The microtuber discs were then allowed to grow on root and shoot regeneration medium. These petri dishes were kept under controlled environment and plantlets with well-developed roots (with rooting efficiency of 73%) were propagated for further experimental analysis.

Transgenic lines selection and expression analyses

Transgenic lines were first screened for positive selection by growing plants on selective medium that contains kanamycin. Later, we used PCR strategy to confirm positive transgenic lines through amplification by *NptII* and gene-specific primer pair and genomic DNA from each line was extracted and used as template. After identification of positive plants, we extracted total RNA by using TRIzol reagent (Sangon, Biotech (Shanghai), Co. Ltd.), for expression analysis of transgenic lines. The expression analysis was carried out through qRT-PCR using and potato *ef1α* gene was used as internal standard control. The qPCR instrument (Bio-Rad, CFX connect™ Real-Time System) was used to perform qRT-PCR reaction and gene expression levels were calculated using a comparative Ct method.

Expression and abiotic stress tolerance assay in transformed *E. coli* cells

To express recombinant protein, we first designed specific primers with restriction enzyme cutting sites (*NdeI* and *HindIII*). After successful PCR amplification and enzyme digestion, the product was ligated with recombinant protein expression vector pET28a. Later, we transformed *E. coli* BL21 (DH3) cells containing recombinant plasmid (pET28a + *StWRKY2*) and pET28a vector alone (control) through electroporation using standard protocol. The culture was grown on selective medium and allowed to grow overnight and subsequently screened to choose the positive clones. The transformants were induced for 8 h at 37°C using 1 m*M* of IPTG (isopropyl b-D-1-thiogalactopyranoside) to express *StWRKY2* recombinant protein. The protein samples were loaded in 12.5% (w/v) SDS-PAGE gel using standard apparatus (Bio-Rad, USA).

In order to examine that recombinant protein expression of *StWRKY2* could enhance the tolerance of *E. coli* cells for different abiotic stress treatments, these transformed cells were grown on solid medium contains: (6% w/v NaCl) for salt stress treatment, (6% w/v PEG or 6% w/v Mannitol) as induced-drought stress treatment, transformed cells grown on solid medium and frozen in liquid nitrogen then thawed for 15 min at 37°C for cold stress treatment, and finally cells were subjected to heat stress by incubation at 50°C for defined time points [10, 20, 30, 40 and 50 min]. The *E. coli* cells were transformed with recombinant plasmid (pET28a + *StWRKY2*) as well as empty vector (pET28a) as control to subsequently perform abiotic stress assays. Initially, the cell cultures were adjusted to an $O.D_{600}$ of 0.6 and subsequently induced by adding 1 m*M* IPTG. Later, the *E. coli* cells were subjected to aforementioned stresses in LB liquid medium and changes in $O.D_{600}$ values were recorded every two hours for 18 h to compare and analyze effects on the growth of recombinant and control cells as described in previous reports (Zhou *et al*. 2017). Three independent experiments were performed and each time cells were subjected to stress in fresh LB medium to confirm the results.

Abiotic stress tolerance assays in transgenic potato and wild type plants

To investigate whether expression of *StWRKY2* play important roles in regulating hormone signaling, we subjected overexpression (OE) and wild type (WT) plants with four important phytohormones namely, salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA). The plant hormones were first adjusted to following concentrations: 2 *m*M SA, 100 *μM* JA, 100 *μ*M ET and 100 *μ*M ABA. Four to six weeks old plants were sprayed with adjusted hormones and samples were collected in frozen liquid nitrogen at specified time points.

In order to elucidate the role of *StWRKY2* in drought tolerance, we conducted three independent experiments by exposing pot grown OE and WT plants for drought stress for 15 days. The first experiment aimed to determine the effect of drought on whole plant, so we conducted survival bioassay and plants were allowed to normally grow in growth chambers and later exposed to drought stress by water deprivation for 15 days. The ratio of plants survival between wild-type and over-expression plants was calculated and plants that could not be recovered even after re-watering were considered dead. To further confirm our results, we performed the second experiment through detached-leaf assay in which leaves were first detached from plants and then placed on open petri dishes with their abaxial side up. Finally, initial and final weights of leaves were recorded to calculate the percent water loss at predetermined time points. Moreover, the stomatal conductance was monitored as the third experiment using an AP4 porometer to determine the effect of drought on stomatal closure, as previously described (Sun *et al*. 2013).

Meanwhile, plants were grown under two water conditions: for drought stress; plants were grown under 25% field capacity (FC), for normal conditions; plants were grown under 100% FC. The field capacity was determined according to the gravimetric method as previously described (Junker *et al*. 2015), which consists on the difference between the wet soil after saturation and free drainage, and the weight of the dry soil. Maintenance of the water treatments was made by daily weighing of the pots replacing the water lost by transpiration using a precision scale until specific field capacities were achieved. In plants grown under these two water conditions that represent field conditions, we determined changes in some stress-related biochemical markers (soluble sugars, proline, hydrogen peroxide H_2O_2 and malondialdehyde MDA). In order to examine the oxidative tolerance, we sprayed leaves of three individual plants both from wild-type and overexpression lines with 100 μM of paraquat. Later, we compared ROS production in OE and WT plants using DAB staining as described in previous methods (Liou and Stroz 2015). Further, the antioxidant enzyme activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in the leaves were determined as previously described (Zhang *et al*. 2015).

Biotic stress assay using *P. infestans* inoculations

For biotic stress assay, evenly grown leaves were first detached both from WT and OE plants and placed inside the inoculation tray. For efficient inoculations, we selected two aggressive *P. infestans* isolates "99183 and 88069" and isolates were re-cultured before starting our experiments. About 3–4 mm plugs were prepared in distilled water and used for inoculation. Leaves were kept under control environment: 16-h photoperiod, 20°C, 100% humidity to facilitate the infection process. We observed the disease symptoms on everyday basis and finally six days later photographs were taken to show comparison of disease phenotype between WT and OE lines. This assay was repeated three times with same temperature, light and humidity conditions. Later, the area under disease was calculated (in cm^2) by recording the size of disease lesion (using digital vernier caliper) on the surface of leaves.

Transactivation activity assay in yeast cells

The transactivation analysis in yeast cells was performed as described in previous report (Wang *et al*. 2012). Briefly, the plasmid (pGBKT7 + *StWRKY2*) was transformed into the yeast strain AH109 (Clontech). The empty pGBKT7 (BD) vector was used as negative control, while pGBKT7-StNAC26 was used as positive control. The transactivation activity was estimated depending on the growth on SD/-Leu/-Trp and SD/-Leu/-Trp/-His.

Statistical analysis

All experiments were performed in three technical and three biological replicates and values are presented as the mean ± SD. For data analysis, we used SAS version 9.1. Means were compared using Duncan's multiple range test to determine the least significant difference among means at the significance level $P < 0.05$.

Results

Identification and phylogenetic analysis of the *StWRKY2* gene

Based on previous microarray results, a uniquely expressed disease-responsive potato WRKY gene (EU056914, https://www.ncbi.nlm.nih.gov/) was identified. We confirmed the complete ORF within cDNA sequence of *StWRKY2* using ORF Finder (https://www.dna20.com/toolbox/ORFFinder.html). Later, we successfully designed the gene amplification primers and clone the *StWRKY2* of size 1065 bp. Our BLAST search using potato genome database (http://solgenomics.net/) revealed its location on chromosome number 01 and its protein consist of 375 amino acids with a predicted molecular weight of approx. 39.85 kDa. Further conserved domain (CDD) search showed that it contains a single WRKY-domain (WD) (Fig. 1). Phylogenetic analysis of *StWRKY2* with WRKY TFs from other plant species such as tomato, cotton, tobacco, Arabidopsis and rice revealed that it is structurally closely related to WRKY group II family proteins (Fig. 1). To explore the functional role of *StWRKY2* in plants, recombinant plasmid constitutively overexpressing *StWRKY2* (35S:*StWRKY2*) was introduced into cultivated potato (cv. E-potato 3, E3). Initially, we screened the transgenic *StWRKY2*-overexpression (OE) plants on selection medium containing kanamycin and finally through PCR using its genomic DNA as template. Fourteen independent lines for *StWRKY2* OE were obtained and out of these positive lines, three OE lines (OE2, OE7 and OE8) that show high transcript levels were finally selected in this study to perform experiments.

Subcellular localization, transcriptional activity and promoter analysis of *StWRKY2*

Subcellular location of *StWRKY2* inside plant cells was confirmed by cloning the complete ORF of *StWRKY2* into binary vector PBI121-GFP aligned with *35S* promoter which is widely used for constitutive expression. The resulting plasmid (35S:*StWRKY2*-GFP) was then injected into tobacco leaves. Consequently, we found that *StWRKY2*-GFP fusion protein was exclusively located inside the cell nucleus (Fig. 2a). On the other hand, the control GFP (*35S*: GFP) was distributed both in the cytoplasm and the nucleus (Fig. 2a), implying that *StWRKY2* is a nuclear protein.

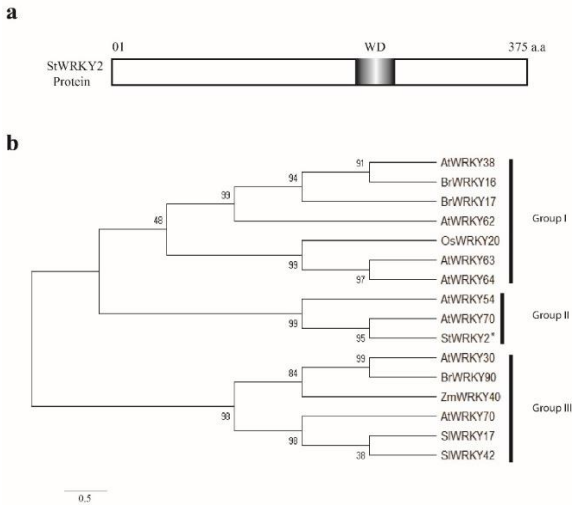

Fig. 1: Conserved domain and phylogenetic tree analysis of *StWRKY2* with other plant WRKY TFs from different species. **a** *StWRKY2* protein length and conserved WRKY-domain (WD) located at the N-terminus. **b** Amino acid sequences of known WRKY TFs belonging to different groups were utilized to construct phylogenetic tree for *StWRKY2* using neignbour-joining method. At, *Arabidopsis thalaiana*; Br, *Brassica rapa*; Os, *Oryza sativa*; St, *Solanum tuberosum*; Zm, *Zea mays*; Sl, *Solanum lycopersicum*

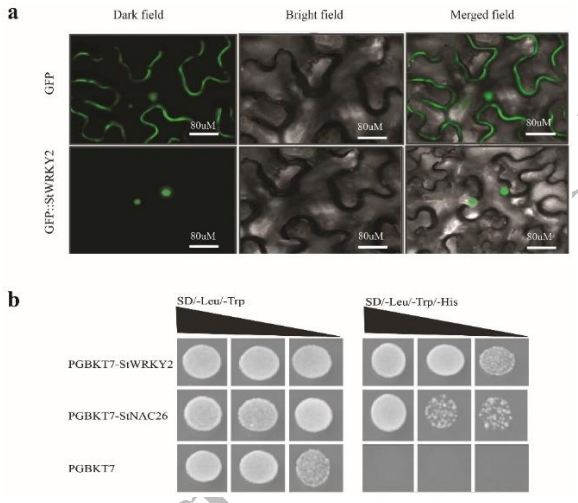

Fig. 2: Subcellular localization and transactivation assay. **a** Subcellular localization of *StWRKY2* was observed in tobacco leaves. Confocal microscope was used to observe signal from cells transformed with GFP alone (control) as well as signals from *StWRKY2*: GFP transformed cells. Bar is 80 μm. **b** Transactivation activity of *StWRKY2* was analyzed using yeast strain AH109. PGBKT7-StNAC26 was used as positive control, whereas PGBKT7 alone was used as negative control. Transformants were incubated on selective medium for transactivation activity

To determine whether *StWRKY2* possess transcriptional activity, we used yeast expression system. Yeast strain AH109 was transformed with fusion plasmids pGBKT7-*StWRKY2* and pGBKT7-StNAC26 (positive control) while an empty vector pGBKT7 was used as negative control. As shown in Fig. 2b, the yeast cells transformed with pGBKT7-*StWRKY2* and pGBKT7-StNAC26 grew well on selective His-medium. On the contrary, yeast cells transformed with empty plasmids could only survive on SD/-Trp medium. These results indicated that the *StWRKY2* has obvious transcription activity in yeast cells.

For promoter analysis, we examined 1.5-kb genomic regions upstream of the transcriptional start of the *StWRKY2* gene using PLACE database. A few important stress-responsive *cis*-acting DNA regulatory elements were found in the *StWRKY2* promoter region including the pathogen responsive related (WRKY element, W-box), ABA-responsive element (ABRE), drought-responsive related (MYB element), dehydration responsive element/C-repeat (DRE/CRT complex), phytohormone SA-responsive element, and the guard cell-specific related (DOF core element), details of which are presented in Table 1.

Expression analysis of *StWRKY2* under various stress conditions and signaling hormones

To examine whether *StWRKY2* is involved in various stress pathways and hormone signaling, we measured its expression pattern under abiotic factors and under various hormones treatments. For abiotic stress conditions, we exposed the plants to drought, salt, cold and heat, whereas SA, JA, ABA and ET were used for hormone treatments. Transcription analysis revealed that *StWRKY2* significantly induced in response to different stress conditions. Under drought stress, *StWRKY2* expression increased after 3, 6, 9, and 12 h, approximately up to 2, 5, 9 and 16-folds, respectively compared with control (non-stressed plants). In the same manner, *StWRKY2* expression was induced under salt stress up to a maximum of 5-folds after 12 h of NaCl treatment compared to control (Fig. 3). Furthermore, potato plants when exposed to heat stress (40°C), the *StWRKY2* transcripts reached to a maximum of 9-fold after 12 h of heat treatment. Cold stress (4°C) resulted in a quick induction of *StWRKY2* transcript within 1 h reaching at maximum after 6 h and then declined (Fig. 3).

Regarding phytohormones treatments, the *StWRKY2* gradually up-regulated in response to SA (100 μM) approximately 3-fold after 2 h and reached its maximum of approx. 16-folds after 24 h of SA treatment. For transcript induction by ABA (100 μM) treatment, we observed a maximum of 2.5-fold increase in *StWRKY2* mRNA accumulation. In case of ET, the *StWRKY2* transcripts initially accumulated up to 1.6-folds within 2 h of treatment and then sharply decreased up to 0.2 and 0.4-folds after 12 h and 24 h, respectively (Fig. 3). In contrast to all other phytohormones, JA (100 μM) mainly decreased the endogenous mRNA level of *StWRKY2* (Fig. 3). These results indicated that *StWRKY2* could respond to wide range of stress and hormone conditions although at different fold levels, implying that *StWRKY2* could be involved in various stress pathways and hormone signaling.

Table 1: Putative cis-elements in the promoter region of *StWRKY2*

Cis-element	Sequence (strand)	Number	Function
MYB	CACCTAAC TTC (+/-)	2	dehydration-responsive
ABRE	GACACGTGGC TTC (+/-)	6	ABA-responsive
MBS	CAACTG TTC (+/-)	1	MYB binding site
W-box	TGAC TTC (+/-)	4	SA, wounding, pathogen
Dof core	AAAG TTC (+/-)	2	oxidative stress
DPBF core	ACACACG TTC (+/-)	1	ABA responsive
GATABOX	GATA TTC (+/-)	6	light responsive
DRE/CRT	ACACACG/ CNAACAC TTC (+/-)	1	ABA, dehydration
MYC consensus	CANNTG TTC (+/-)	5	dehydration responsive
TAAAG motif	TAAAG TTC (+/-)	2	SA, oxidative stress
GT-1 consensus	GRWAAA TTC (+/-)	1	light responsive, SA
CACTFTPPCA1	YACT TTC (+/-)	1	mesophyll-specific
HSE	AAAAAATTTC (+/-)	4	heat stress responsive
CAAT-box	CAAT (+/-)	1	common cis-elements
TC-rich repeats	G/A/TTTTCTTA/C/C/A (+/-)	3	defense and stress responsiveness

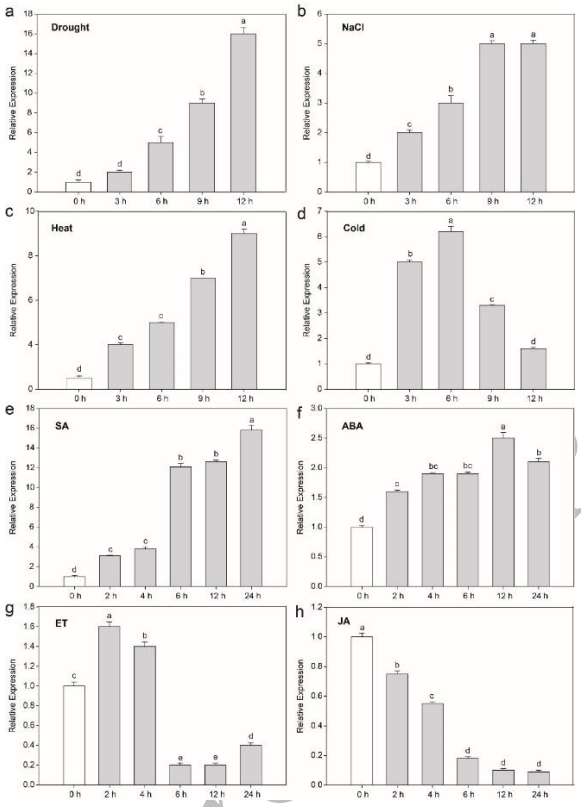

Fig. 3: Expression levels of StWRK2 in potato leaves under phytohormones and different stress conditions. The leaves of 6-weeks old plants were used for RNA extraction in wild-type plants after treatment with **a** drought; **b** 200 m*M* NaCl; **c** 40°C heat; **d** 4°C cold; **e** 2 mM SA; **f** 100 μM ABA; **g** 100 μM ET and *h* 100 μM JA. All samples were collected at the indicated time points ('h' refer to hours after treatment) from three biological replicates. Different letters indicate significant differences at $P < 0.05$ between the stress treatment and the 0h control. Actin gene was used as an internal control in the qRT-PCR

Expression of *StWRKY2* in *E. coli* and its effect on growth of transformed cells under multiple stresses

To check the response of multiple environmental factors, a cDNA sequence of *StWRKY2* was cloned in pET28a vector and then the recombinant plasmid (pET28a-*StWRKY2*) was transformed and expressed into *E. coli*. The protein expression was detected by performing SDS-PAGE and result showed strong induction of a protein of predicted size in pET28a: *StWRKY2* transformed cells; conversely, we did not detect target protein expression in un-induced cells and empty vector control (Fig. 4a). To comprehend the critical role of *StWRKY2* protein in various stress responses, we analyzed the growth pattern of *E. coli* transformed with plasmid (pET28a: *StWRKY2*) or empty vector under various stress factors including mannitol, PEG, NaCl, heat and cold treatments.

No significant difference was recorded in the growth of pET28a alone or pET28a transformed with *StWRKY2* under optimal conditions. However, under all stress conditions the transformed *E. coli* cells exhibited significantly faster growth whereas the growth of control cells was inhibited by NaCl, PEG, mannitol, cold and heat treatments (Fig. 4). The growth of all cells was monitored spectrophotometrically at an optical density of 600 nm (OD_{600}). These findings suggested that expression of *StWRKY2* protein significantly improves tolerance of *E. coli* cells against various stress factors, and thus lead us to further study its possible function as multiple stress responsive WRKY transcription factor in plants.

Growth, physiology and enhanced drought tolerance of transgenic plants

In order to determine whether *StWRKY2* overexpression could elevate tolerance level of transgenic plants against drought, the OE and WT plants were subjected to drought stress for 15 days. As expected, WT plants started to wilt just after 10 days without water but not transgenic plants. After 15 days without water, WT plants showed more severe wilting symptoms. Interestingly, most of *StWRKY2* OE plants remained turgid and showing substantial tolerance even after 15 days of drought (Fig. 5a). Additionally, even after watering was resumed to normal, WT plants could not be recovered that showed clear signs of severe drought stress.

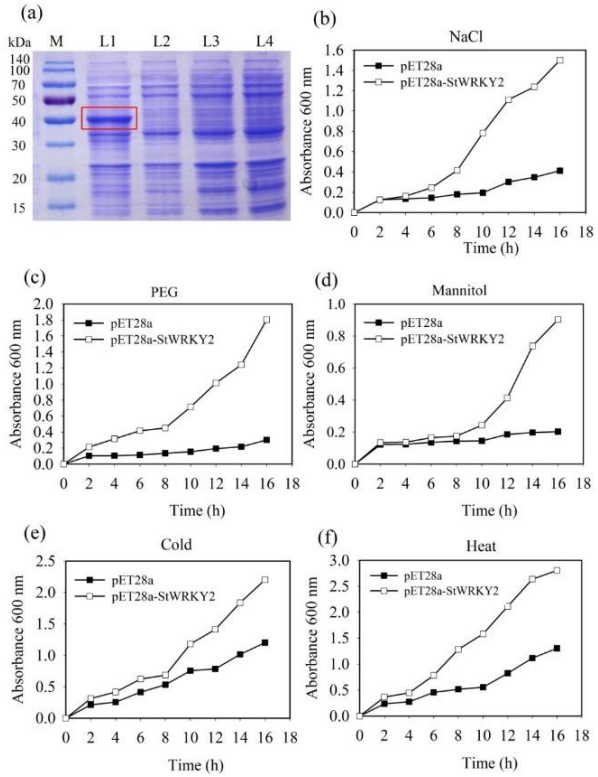

Fig. 4: Over-expression of *StWRKY2* improves tolerance to various stresses in *E. coli*. **a** SDS-PAGE (12.5%) analysis of *StWRKY2* protein expression in *E. coli* BL21 (comassie blue staining); where Lane M; protein marker (kDa), L1; whole cells lysate of BL21 *E. coli* cells containing the plasmid pET28a: *StWRKY2* obtained at 8 h post-induced with 1 mM IPTG, L2; whole cells lysate of non-induced BL21 *E. coli* cells containing the plasmid pET28a: *StWRKY2*, L3; whole cell lysate of BL21 *E. coli* cells containing the empty vector pET28a without IPTG induction, L4; whole lysate of BL21 *E. coli* cells containing the empty vector pET28 obtained at 8 h post-induction with 1 mM IPTG. Growth analysis of *E. coli* carrying *StWRKY2* gene was carried on LB liquid medium with different supplements and stresses: **b** NaCl (6%) **c** PEG (6%) **d** Mannitol (6%) **e** Cold (frozen in liquid nitrogen for 1 min) and **f** Heat (50°C) OD_{600} was recorded at 2 h interval up to 18 h and mean values are represented in graph

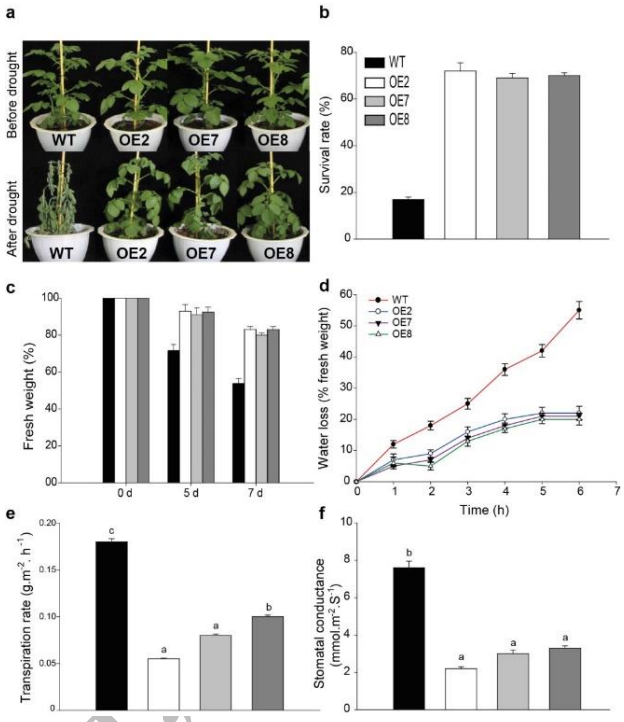

Fig. 5: Transgenic plants overexpressing *StWRKY2* are tolerant to drought stress conditions. **a** Eight-week-old potato plants were deprived of water for 15 days and then water was resumed **b** Survival rates of wild type and transgenic potato plants overexpressing *StWRKY2* under drought stress conditions. Plants were scored for viability. **c** Fresh weight of plants from transgenic potato and wild type under drought stress conditions. Fresh weights of whole plants from transgenic and wild type potato were measured. **d** The kinetics of water loss in detached leaves from wild type and transgenic potato plants. Water loss is presented as the percentage of weight loss versus initial fresh weight. **e** Transpiration rate of the detached leaves were detected after leaves were placed on a filter paper and exposed under white florescent light for 5 h and **f** Stomatal conductance of leaves from WT and transgenic lines were measured. Three independent experiments were performed, and values represent the means ± SE of three independent experiments

Survival rate and plant growth was determined one week after the resumption of watering both for stressed and non-stressed control plants. Around 70% of *StWRKY2* OE potato plants survived well after the period of drought stress compared with 17% of WT plants (Fig. 5b). Fresh weight of WT and *StWRKY2* OE potato plants was also measured before and after drought stress. No significant difference was observed between wild-type and transgenic lines before drought stress treatment. However, five days after water deprivation, water content of WT plants was decreased by 27% whereas only 6–8% decrease was observed for *StWRKY2* OE lines. After 7 days of water deprivation, water content of WT and OE plants was decreased by approximately 44 and 18%, respectively (Fig. 5c). Overall, these results clearly demonstrate that expression of *StWRKY2* enhanced the drought tolerance of transgenic potato as compared to WT plants most likely through improving the drought adaptive mechanisms.

Based on our results that demonstrate the positive regulation of drought stress by *StWRKY2* was further confirmed by *in vitro* detached-leaf water loss assay. We detached the leaves from similar branches from bottom and measure their fresh weights to calculate the water loss rate. Fresh weight of *StWRKY2* OE leaves was decreased rather slowly up to 21% compared with the fresh weight of WT leaves that rapidly decreased up to 56% after the drought stress (Fig. 5d). Additionally, *in vitro* drought test showed that stomatal conductance and transpiration rate of OE plants was significantly reduced compare to WT plants (Fig. 5e and f). These findings imply that *StWRKY2* play

significant role under drought stress by regulating stomatal movements and affecting stomatal size to reduce water loss thus it contributes significantly to improve the overall adaptive mechanisms of transgenic plants.

Overexpression of *StWRKY2* enhanced the ROS-scavenging ability of transgenic plants under drought conditions

To divulge whether *StWRKY2* modulates the production of ROS under drought conditions, the oxidative burst was first observed on detached leaves using DAB staining under normal and drought stress conditions (by exposing leaves to paraquat). No significant difference was seen between transgenic lines and WT leaves under normal conditions. After exposure to drought stress, DAB staining results showed more browning of WT leaves than leaves from OE lines (Fig. 6b). Moreover, when plants were exposed for 15 days of drought stress, higher H_2O_2 accumulation in WT was observed in comparison with OE lines (Fig. 6a), which clearly indicated that overexpression of *StWRKY2* in transgenic potato enhanced the ROS-scavenging ability under drought conditions.

For further confirmation, oxidative stress related plant biomolecules (soluble sugars, proline and malonaldehyde), were subsequently measured under normal and drought stress conditions. No significant differences were recorded under normal conditions. However, by no surprise MDA level of OE lines was 25–33% lower than WT in response to drought stress treatments (Fig. 6c). This reduction of MDA content suggested that *StWRKY2* can alleviate cell membrane damage after exposure to drought stress. In contrast, the proline concentration of OE lines was 2-folds higher than WT (Fig. 6d). In the same manner, the soluble sugars content was significantly increased in OE lines compared to WT plants under drought stress (Fig. 6e). Furthermore, the total activity of antioxidant enzymes including SOD, CAT and POD were measured under normal and drought stress conditions. Under normal conditions, no significant differences were observed in SOD, CAT and POD activity between wild-type and transgenic lines. Conversely, transgenic lines displayed markedly higher levels of SOD, CAT and POD activity, which was increased up to 1.5, 2 and 1.6-folds higher, respectively, compared with WT plants under drought stress (Fig. 6f, g and h). These results indicated that expression of *StWRKY2* in potato has led to improve its ROS scavenging ability and thus revealed the mechanism of resistance in transgenic plants under drought stress.

Expression of *StWRKY2* enhanced resistance against *P. infestans* thereby regulating the expression of pathogenesis-related *(PR)* genes in transgenic potato

One of the most destructive pathogens of potato is *P. infestans* that causes late blight disease. To test whether the overexpression of *StWRKY2* could also confer resistance

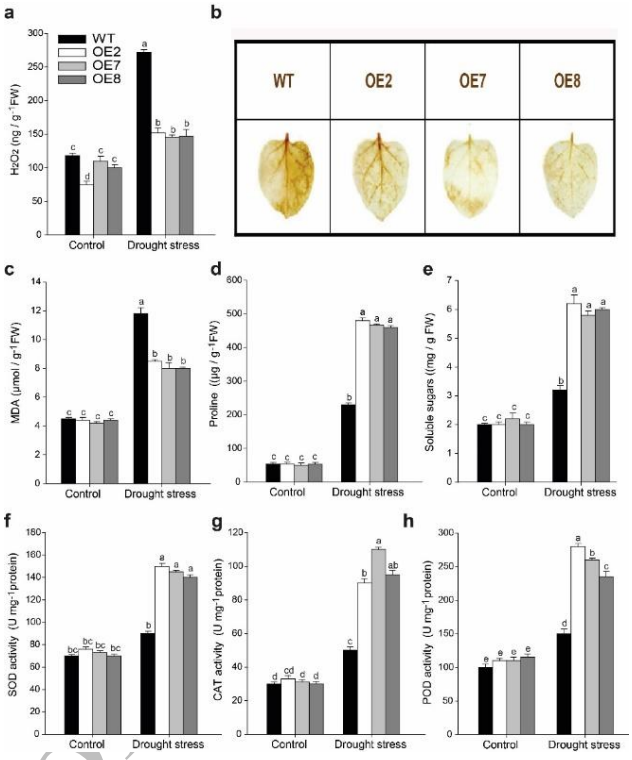

Fig. 6: Biochemical characterization of transgenic potato plants overexpressing *StWRKY2* and wild type plants under normal and drought stress conditions. **a** H_2O_2 content. **b** oxidative stress assay was conducted by spraying paraquat on the leaves of WT and transgenic plants. Phenotype of leaves after DAB staining (for 24 h) were recorded **c** MDA content. **d** Proline accumulation. **e** Soluble sugar content. Antioxidant enzyme activity in WT and *StWRKY2* OE plants under normal and drought stress conditions *i.e.*, activities of **f** SOD, **g** POD, **h** CAT, respectively. Values represent the means ± SE of three independent experiments. Different letters indicate significant differences at $P < 0.05$ between the transgenic and wild type lines under drought stress

to *P. infestans* in transgenic plants, detached leaves of WT and OE lines were inoculated with mix isolates of *P. infestans*. Six days post inoculation, WT leaves showed pronounced disease symptoms (*i.e.*, water-soaking areas showing the presence of heavy oomycete hyphae), whereas leaves from OE lines showed significantly enhanced resistance phenotype against *P. infestans* (Fig. 7a). To quantify the disease progress, disease lesion size was observed and calculated on the leaves of WT and OE lines. The results showed that disease lesion covered approximately 58–64% of WT leaf area; whereas a remarkably reduced disease lesion size was observed on leaves from three transgenic lines (Fig. 7b). This data clearly shows that overexpression of *StWRKY2* has significantly inhibited the disease progression and thus plays a crucial role for enhanced resistance in *StWRKY2*-transgenic potato.

Various pathogen-related (*PR*) genes including *PR2*,

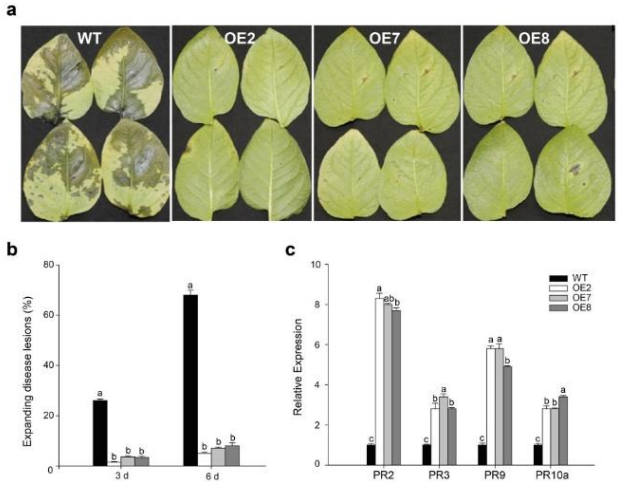

Fig. 7: *StWRKY2* overexpression confers resistance towards biotic stress. **a** *StWRKY2* OE plants exhibited enhanced resistance to late blight. Test plants were inoculated with *Phytophthora infestans* and then kept at 20ºC with a photoperiod of 16 h. The photograph was taken after six days; **b** Expanding disease lesion rate of *Phytophthora infestans* was observed and calculated (in mm) on the surfaces of leaves using digital Vernier caliper and scored as percentage. **c** Relative expression levels of *PR2*, *PR3*, *PR9* and *PR10a* in WT and transgenic lines following infection

PR3, *PR9* and *PR10a*, are known as resistance/defense marker genes. Promoter of these genes contains WRKY DNA-binding proteins that recognize functional W-box (TTGAC[C/T]). In order to investigate whether PR genes were induced upon *Phytophthora* infection, we measured the expression level of *PR2*, *PR3*, *PR9* and *PR10a* in WT and *StWRKY2*-OE lines following infection. Interestingly, our results revealed that 24 h post-inoculation, mRNA levels of *PR2*, *PR3*, *PR9* and *PR10a* in OE lines reached to highest levels of upto 8, 3.6, 5.5 and 3.8-folds, respectively as compared to WT plants (Fig. 7c). This result indicates that overexpression of *StWRKY2* in transgenic potato enhanced its resistance to late blight via up-regulating the *PR* genes, which is consistent with several earlier reports (Shahzad *et al*. 2016; Ewas *et al*. 2017a; Liu *et al*. 2018).

Discussion

A Major group of proteins are known as transcription factors, which regulate the expression of target genes by binding to *cis*-elements in the promoter regions of upstream or downstream genes (Sheshadri *et al*. 2016). Plant transcription factors serve as gene regulators and engage in most of biological processes, including metabolism, growth, development, biotic and abiotic stresses resistance. Increasing evidence indicates the modulation of these stresses by multiple phytohormone signaling pathways such as ABA, SA, JA, ET as well as ROS (Fujita *et al*. 2011; Sewelam *et al*. 2016). With the discovery of WRKY gene family at the end of the 19th century as transcription factors, the mechanisms of multiple physiological and biological processes were revealed (Wu *et al*. 2019). Interestingly, our results showed that *StWRKY2* expression changed under abiotic stress conditions and various plant hormones and this may be attributed to the presence of putative stress-responsive *cis*-elements (*i.e*., MBS, MYB, MYC, HSE, ABRE, TGA1 and DPBF1, 2) in the promoter region of *StWRKY2*. The *cis*-elements are the core elements for initiating appropriate defense response (Makhloufi *et al*. 2014). We found few important *cis*-elements in promoter region which clearly indicate that *StWRKY2* could bind and regulate the expression of downstream stress-related genes. Promoter analysis of *StWRKY2* also revealed some biotic stress-response elements including W-Box/Box-W1 and TC-rich elements. Interestingly, pathogenesis-related (*PR*) proteins (*PR2*, *PR3*, *PR9* and *PR10a*), which are known as resistance/defense marker genes (Shahzad *et al*. 2016), were markedly up-regulated in *StWRKY2* OE plants and further revealed the cause of elevated resistance to late blight.

Drought is sort of oxidative stress in plants, while MDA, proline and soluble sugars are osmoprotectants and important index of plant oxidative stresses (Loukehaich *et al*. 2012; Ewas *et al*. 2016; Nounjan *et al*. 2018; Lu *et al*. 2019; Guo *et al*. 2019; Sattar *et al*. 2019b). Here in this study, proline and soluble sugars contents of *StWRKY2* OE lines were higher than WT, while the MDA contents were reduced in OE plants than WT under drought stress conditions, which consequently enhanced the drought tolerance of all three OE lines. These results are in consistence with corresponding tolerance reported earlier in potato (Wang *et al*. 2017), tomato (Loukehaich *et al*. 2012; Ewas *et al*. 2017b), Arabidopsis (Yu *et al*. 2016), tobacco (Ziaf *et al*. 2011), and rice (Cui *et al*. 2016). Several studies have implicated antioxidant enzymes, SA and ABA in abiotic stress responses (Iglesias *et al*. 2011; Sharma *et al*. 2012). The results of present study showed that the activity of antioxidant enzymes (SOD, POD and CAT) was also significantly increased under drought stress. These results are consistent with the predetermined fact that antioxidant enzymes activity is increasing under drought stress conditions in many other crops such as tomato (Murshed *et al*. 2013), faba bean (Siddiqui *et al*. 2015) and rice (Wang *et al*. 2019). Our results indicated the positive role of *StWRKY2* in stomatal movement. Previous studies demonstrated a crosstalk between drought and ROS signaling during stress tolerance, however, the role of antioxidants and stomatal movement in adaptive abiotic stress response has been well reported (Bashandy *et al*. 2010; Sharma *et al*. 2012). Further DAB staining and H_2O_2 analysis also revealed that OE lines were more tolerant thereby protecting the plants from oxidative damage under drought stress.

Conclusion

We investigated the role of a potato WRKY gene, *StWRKY2*, in response to various environmental stresses. In *E. coli*, the expression of *StWRKY2* imparts tolerance to

wide-range of different stresses. The transgenic potato plants expressing *StWRKY2* also exhibited better growth under drought stress and showed more resistance to *P. infestans* by activating the distinctive physio-biochemical and molecular mechanisms in potato. This study further paves the way for utilizing *StWRKY2* gene that could be used as candidate for future breeding programs aim to improve broad-spectrum resistance in plants especially in solanaceous crops.

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