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Overexpression of potato transcription factor (*StWRKY1*) conferred resistance to *Phytophthora infestans* and improved tolerance to water stress

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Abstract

Potato (*Solanum tuberosum*) late blight caused by oomycete *Phytophthora infestans (Pi)* is highly destructive to potato yield and cost huge losses each year. Regulation of a network of transcription machinery, controlled by transcription factors (TF's), is required to overcome the susceptibility. WRKY TF's are known to regulate transcription machinery upon biotic and abiotic stresses in different crop plants. We cloned and characterized a WRKY gene, *StWRKY1*, from potato cDNA synthesized from *Pi* infested leaves. *StWRKY1* protein localized typically in the nucleus. Overexpression (OE) of *StWRKY1* positively regulates *Pi* resistance as well as drought tolerance in transgenic potato. The elevated resistance in OE lines was co-related with higher accumulation of pathogenesis-related (PR) genes as compared to untransformed control plants. Interestingly, increased susceptibility of co-suppression (CSP) plants was associated with down regulation of PR genes expression. Moreover, transgenic lines overexpressing *StWRKY1* showed tolerance in terms of less rate of water loss, during dehydration assay. Importantly, expression of *StWRKY1* was upregulated upon treatment with plant hormones, suggesting its involvement in basal signal transduction pathway. Overall, our findings provided evidence that *StWRKY1* positively regulate biotic and abiotic stress resistance thereby modulating plant basal defense networks, thus play a significant role for crop improvement.

Keywords: Abiotic stress, biotic stress, overexpression, PR, potato, transcription factor.
Abbreviations: PR_pathogenesis-related; mRNA_messenger RNA; CaMV35S_cauliflower mosaic virus; ORF_open reading frame; qRT-PCR_quantitative real time PCR; DPI_ days post inoculation; GFP_green fluorescent protein; RFP_red fluorescent protein.

Introduction

Potato is the world's third-largest food crop for consumption. Just over two thirds of the global production is eaten directly by humans with the rest being fed to animals or used to produce starch or processed food. It suffers from many pests and diseases among which late blight, caused by *Phytophthora infestans (Pi)*, is the worst (Haverkort et al., 2009). Substantial advancement has been made to understand the systems by which plants identify and safeguard themselves against pathogen attack. These engross the characterization of plant disease resistance genes that recognize particular strains of pathogens (Dangl and Jones, 2001), followed by components involved in the signal transduction pathways thereby pathogen recognition to brisk up defense responses (Glazebrook, 2001). In addition, endogenous plant signaling molecules that include salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) are key mediators in basal defense mechanisms (Thomma et al., 2001; Asselbergh et al., 2008; Mauch-Mani and Mauch, 2005). Transcription factors play key functions in signal transduction pathways to activate or suppress defense gene expression. They directly regulate downstream target gene expression by binding to specific elements (cis-elements) in the promoters. Responses and a great deal of adaptations require differential gene expression that is regulated by specific transcription factors (Jiang et al., 2014a). More than 1500 genes encoding transcription factors have been described in the *Arabidopsis* genome such as NAC, b-ZIP, Myb/c, WRKY and AP2/ERF (Xu et al., 2011). The widely recognized WRKY family proteins that are defined by the presence of the WRKY domain are well known among them. This contains the amino acid core sequence WRKYGQK that is conserved in all WRKY domains so far analysed. WRKY DNA-binding proteins recognize functional W-box (TTGAC[C/T]) present in promoters of various pathogen-related (PR) genes and can be induced by SA, JA, ABA and ET treatments (Pape et al., 2010; Lim et al., 2011). Most PR genes offer antimicrobial activities marked by hydrolytic reaction on pathogen cell walls, respond to toxicity or found to be involved in modulating defense signaling (Van Loon et al., 2006). The fundamental roles of PR proteins belonging to group 2 and 3 against pathogens, has been clearly demonstrated to substantiate a β-1,3-glucanase and chitinase activity respectively (Sabater-Jara et al., 2010). In order to induce effective responses PR-2 can either directly hydrolyze the β-1,3/1,6-glucans within fungal cell walls (Mauch et al., 1988) or release short glucan fragments from pathogen cell walls, that can be perceived by plants and further induce defensive outcomes (Ebel and Cosio, 1994). PR-3 chitinases are linked to degradation of chitin within fungal cell walls (Van

Loon and Van Strien, 1999). The PR-9 is coupled with peroxidase activity (Van Loon et al., 1994), and participate in several defensive tactics including fungal cell wall metabolism and wound healing to further accelerate antimicrobial activity. In addition to their DNA-binding ability, WRKY proteins share other features of transcription factors, such as nuclear localization and transcription activation/repression capability of target genes (Hara et al., 2000). There is a growing evidence of involvement of WRKY factors in modulating basal defense networks and provide effective and immediate responses against both biotic and abiotic stresses. For instance, accumulation of transcripts of a gene from *gossypium hirsutum* (*GhWRKY15)*, were enhanced during the exogenous application of SA, JA and ET signaling compounds. Furthermore, tobacco plants overexpressing *GhWRKY15* exhibit enhanced viral and fungal resistance (Yu et el., 2012). Overexpression of the cotton gene *GhWRKY39-1* enhances resistance to pathogen infection and exhibit tolerance to high salt and oxidative stress (Shi et al., 2014).

Previously *StWRKY1* found to be induced by *Pi* at earlier stage of infection (Dellagi et al., 2000; Machinandiarena et al., 2012). Another study revealed its involvement to reinforce secondary cell walls, by regulating downstream hydroxycinnamic acid amide (HCCA) biosynethetic genes, thus provide significant defence against *Pi* (Yogendra et al., 2015). However, we aim to investigate whether *StWRKY1* transcripts accumulation directly involved to enhance resistance to *Pi*, thus we have generated overexpression transgenic lines to perform disease evaluation test. In this study, we further showed a comprehensive expression analysis of three important PR proteins in relation to late blight disease resistance in *StWRKY1* transgenic lines.

Results

StWRKY1 characterization and overexpression vector construction

The full-length nucleotide sequence of *StWRKY1* TF consist of 1623 bp and it encodes a protein of 541 amino acids with a molecular weight of 58.74 kDa. Sequence analysis shows that *StWRKY1* protein contains a DNA-binding domain (WRKY domain) located at amino acid position 297-353. Our first goal was to generate overexpression plants for disease resistance evaluation. For overexpression vector, complete CDS of *StWRKY1* was fused under control of CaMV35S promoter (Fig. 1a). Microtubers were subsequently infected with *Agrobacterium tumefaciens* containing positive plasmid to obtain overexpression lines.

Subcellular localization and tissue specific expression of StWRKY1

To confirm the subcellular localization of putative transcription factor, we generated a C-terminal translational fusion of *WRKY1* cDNA with the sequence encoding GFP. The plasmid containing CaMV35S:*StWRKY1*:GFP (Fig. 1b), was then injected into tobacco leaves. For positive control, we used ORF of *Ghd7*, a member of transcript family, which was fused in frame with GFP. The recombinant protein exclusively accumulated in the nucleus as expected (Fig. 2). The result suggest that *StWRKY1* protein is localized to the nucleus and support its predicted role in transcriptional regulation. The WRKY genes that are highly expressed in different plant organs often play key roles in plant development, thereby regulating expression of target genes that are involved in some physiological pathways (Pan et al., 2009). The expression level of *StWRKY1* was highly induced in mature leaves followed by stem, flower, root, tuber and young leaves (Fig. 3). Together, our findings demonstrated that transcriptional regulation of *StWRKY1* might play key roles in potato development.

Expression pattern of StWRKY1 by signaling molecules

Phytohormones play pivotal signaling roles in plant-pathogen interactions. We confirmed involvement of *StWRKY1* upon exogenous application of plant hormones, hence linking plant-pathogen interaction and role of *StWRKY1* during the signal transduction pathways. SA has long been known to play a central role in plant defense against pathogens. SA induced the expression of *StWRKY1*, to its maximum of more than 10 folds immediately after 12 h and remain constant until 24 h. In case of ABA, the expression elevated to 3.9 folds at 24 h while slightly decreased after 48 h. The expression level of *StWRKY1* reached to nearly 3.8 and 4.3 folds at 24 h, thereafter expression was greatly reduced both by JA and ET respectively. The details of results are illustrated in Figure 4. Taken together, these results strongly suggest that *StWRKY1* is required to activate the basal defense network mediated by important plant hormones.

Overexpression of StWRKY1 in transgenic potato show enhanced resistance to Phytophthora infestans

Initially putative transgenic *StWRKY1* lines were confirmed through PCR strategy using *NptII* and gene specific primer pairs (Fig. 5a). Of these transgenic lines, three OE lines (referred to as L7, L4, L13), and one CSP line (referred to as L5), were considered for further molecular analysis (Fig. 5b). Selected transgenic lines for *StWRKY1* were subjected to resistant test for mix isolates of *Pi* to confirm the efficient level of resistance. Six days after inoculation, the leaves overexpressing *StWRKY1* showed a clear resistant response to isolate mix at inoculation sites, whereas control leaves showed water-soaking symptoms (Fig. 6a) covered with heavy oomycete hyphae. To quantify the lesions, the length and width of the disease was measured in transgenic and CK leaves, and the data (Fig. 6b) was consistent with what was observed visually. It was obvious that *StWRKY1* inhibited disease development represented by remarkably smaller lesion size compared to the control. Similar disease symptoms results obtained in *Arabidopsis* overexpressing *AtWRKY33* against pathogen *Pseudomonas syringae* (Zheng et al., 2006). Interestingly, CSP plants exhibited more or less similar symptoms of disease lesions as control plants upon inoculation. To further elucidate the underlying mechanism of *Pi* resistance in transgenic lines, we examined the expression pattern of *StWRKY1* during induction period. Relative gene expression data revealed that the transcripts were increased in time dependent manner in OE transgenic lines compared to untransformed control plants (Fig. 6c). On contrary, *StWRKY1* was down regulated in CSP plants during the induction process. These results clearly indicate that the up-regulation of *StWRKY1* transcripts after *Pi* inoculation is vital to provide durable resistance in transgenic potato lines.

Expression analysis of PR genes in transgenic lines

WRKY DNA-binding proteins recognize functional W-box (TTGAC[C/T]) present in promoters of various pathogen-related (PR) genes including PR-2, PR-3 and PR-9. qRT-PCR analysis showed that PR-2 mRNA level reached to much higher level of 23 folds as compared to control, after 48 h of inoculation in OE line L4, while data showed its down

Table 1. Primer sequences used for amplification and expression analysis of *StWRKY1*.

Experiment	Gene	Primer sequence (5` to 3`)
Cloning of *StWRKY-1*	*StWRKY1* F	CG**GGATCC**ATGGACAAAGGATGGGGTCT
	StWRKY1 R	GC**GAGCTCGGTACC**TGTTGTTATTTATTGCTTGAGGGG
Confirmation of transgenic plants	NptII F	GCTATGACTGGGCACAACAG
	NptII R	ATACCGTAAAGCACGAGGAA
Expression analysis using qPCR	Efl α F	ATTGGAAACGGATATGCTCCA
	Efl α R	TCCTTACCTGAACGCCTGTCA
	q*StWRKY1* F	CTCAGGACAACATTCATCACCC
	q*StWRKY1* R	ATGGGGCTGTTGCCGTTAT
	PR2 F	TGATCCGAATCAAGGAGCTT
	PR2 R	TGTCTTGTGTGGCACCAAAT
	PR3 F	GATGATACCGCCCGTAAGAA
	PR3 R	AAGAAACAACACCAGGGCAC
	PR9 F	AAGAAACAACACCAGGGCAC
	PR9 R	TGCCCTCAAGCTGAAGAAAT
GFP vector construct	Attb1	GGGGACAAGTTTGTACAAAAAAGCAGGCT
	Attb2	GGGGACCACTTTGTACAAGAAAGCTGGGT
	StWRKY1-GFP F	AAAAAGCAGGCTTAACCATGGACAAAGGATGGGGTCT
	StWRKY1-GFP R	AGAAAGCTGGGTATGTTGTTATTTATTGCTTGAGGGG
	Ghd7-GFP F	AAAAAGCAGGCTATGGGGATGGCCAATGAGGAGTC
	Ghd7-GFP R	AGAAAGCTGGGTR GAGGAATCCGGCCGCCTTTTTTC

Bold sequence showing restriction sites for gene cloning, while underlined sequences are adopter sites for gateway cloning system.

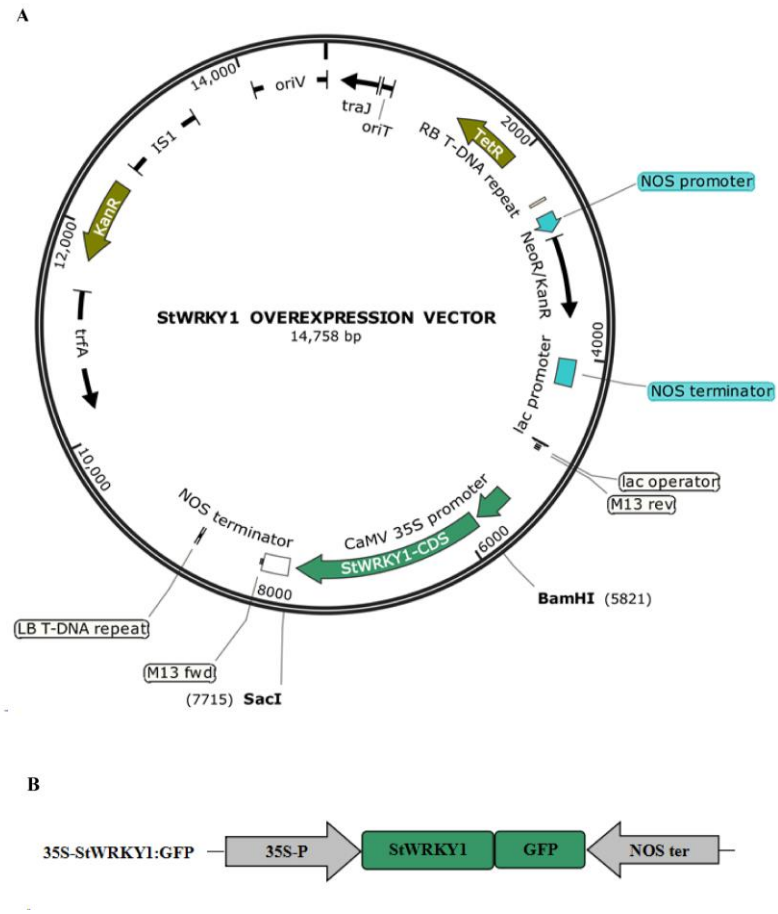

Fig 1. Schematic representation of *StWRKY1* vector constructs. (A) ORF of the *StWRKY1* fused with vector pBI121 to construct *StWRKY1* overexpression vector under the control of CaMV 35S promoter (B) ORF of the *StWRKY1* in frame with GFP was constructed under the control of CaMV 35S promoter for GFP vector.

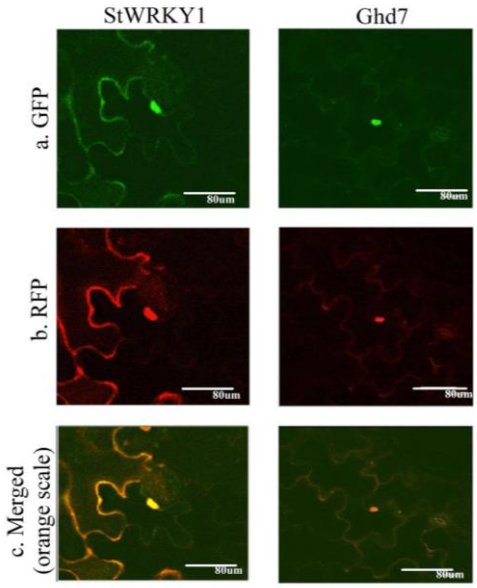

Fig 2. Subcellular localization of potato *StWRKY1*, revealed that GFP signal mainly located in nucleus of the cell, which strongly confirmed by RFP signals fused with GFP. Confocal microscope was used to observe (a) GFP signal inside the cell also (b) RFP signal was detected in both modes fused with GFP in the nucleus as well as in (c) merged orange scale. Ghd7 TF, used as positive control to validate the signal in both modes.

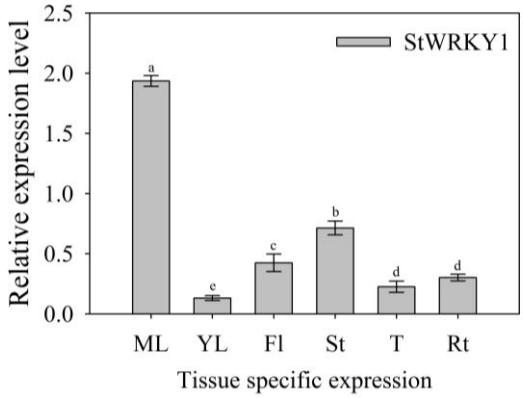

Fig 3. Expression profile of *StWRKY1* in different organs (ML, mature leaf; YL, young leaf; Fl, flower; St, stem; T, tuber; Rt, root) of potato plants showed that, its relative expression was comparatively high in mature leaves followed by stem, flower, root, tuber and young leaf. Vertical bars bearing different letters indicate significant differences at $p < 0.05$.

regulation in CSP line L5. In case of PR-3, the expression level reached to maximum of ~19 folds, exhibited by both OE lines L7 and L4 after 48 h of inoculation, while this level was well maintained in L4 after 72 h. PR-9 expression reached to its peak of ~14 folds in L7 after 72 h compared to control plants. However, the expression of PR-9 was not as rapid and massive as PR-2 and PR-3 in other OE lines. Interestingly, PR-3 and PR-9 genes expression were also down regulated in CSP line L5. The results are given in details in Figure 7. Overall, the data suggest that *StWRKY1* regulate the expression of PR genes in order to provide effective resistance against *Pi*.

Tolerance of overexpression transgenic plants to dehydration assay

In order to investigate the role of *StWRKY1* to modulate plant resistance in water limited conditions, dehydration test was conducted. Detached-leaf assay manifested that OE plants were more tolerant to water limited conditions, as the rate of percent water loss was significantly lower than that of the control plants (Fig. 8). Whereas, co-suppression of *StWRKY1* render the plants more sensitive to water limited conditions. A similar study conducted for a gene cloned from wild soyabean (*Glycine soja; GsWRKY20*) and overexpressed in *Arabidopsis*. Transgenic lines overexpressing *GsWRKY20* showed lower rate of water loss compared to WT during dehydration assay (Luo et al., 2013). A reduced water loss is a major factor contributing to drought tolerance; the leaves of all transgenic lines overexpressing *StWRKY1* showed a slower rate of water loss than those of CK and CSP lines, thus suggesting positive role of *StWRKY1* in drought tolerance.

Discussion

The result of activating a signal transduction pathway is the stimulation or repression of the expression of one or several genes regulated by plant TF's. Therefore, after the perception of the signal by exogenous or endogenous receptors, nuclear proteins including plant TF's have to be imported into the nucleus in order to exert their activity (Vandromme et al.,

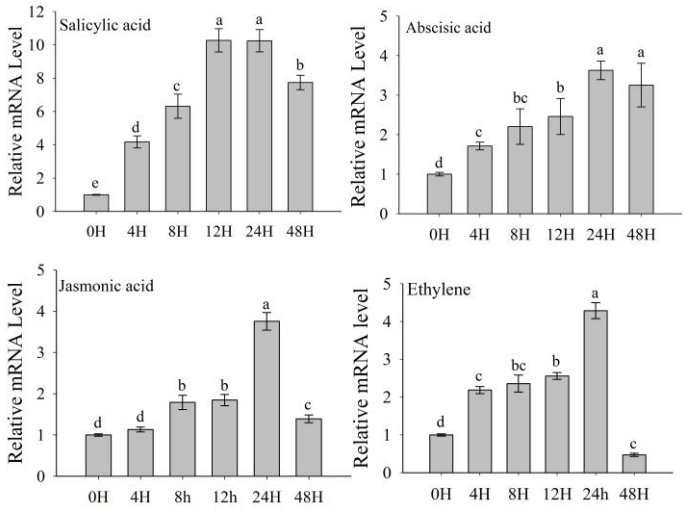

Fig 4. Expression of StWRKY1 in response to various phytohormones. Leaves from 4-6 week old plants were sprayed and samples were immediately frozen in liquid N_2. Relative mRNA level of StWRKY1 in response to four important plant hormones (JA, SA, ET, ABA), were monitored through real time quantitative PCR. Vertical bars bearing different letters indicate significant differences at $p < 0.05$.

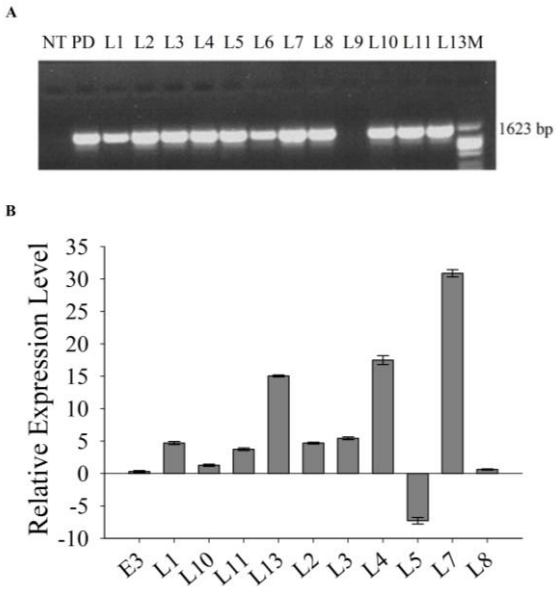

Fig 5. (A) PCR was performed using genomic DNA extracted from 2 weeks old potato leaves to confirm positive transgenic lines. NT, non-transgenic lines; PD, plasmid DNA; L1-L13, transgenic lines; M, 2Kb DNA marker. (B) Expression of StWRKY1 in different transgenic lines relative to control were checked through real time quantitative PCR. Error bars indicate ± SE of means at $p < 0.05$.

1996). Nuclear localization of *StWRKY1,* supported its predicted role in transcriptional regulation. In the past few years, numerous transcription factors that binds to specific DNA sequence have been unveiled, including WRKY factors which binds to wide range of plant defense genes. Transcriptional regulation of these defensive genes by the WRKY factors turned out to be very useful in terms of elevated plant resistance against various pathogens, abiotic stress response and controlling the developmental process (Zhou et al., 2008; Shi et al., 2014). The WRKY genes that are highly expressed in plant organs often play key roles in plant development. According to Ramamoorthy et al. (2008), WRKY genes showed tissue-specific (including young and mature leaves) expression pattern with varied intensities hence suggested divergence in its biological roles in plant development. We collected samples from young and mature leaves in order to observe transcripts pattern of *StWRKY1* in different physiological states. Where young leaf; refers to newly developed leaves prior to stolon development, and mature leaves; refers to leaf samples where plants were actively producing tubers. Transcripts of *StWRKY1* were highly induced in mature leaves, while down regulated in young leaves. A distinct regulation of *StWRKY1* transcripts in young and mature leaves, showing its active involvement at this physiological stage; representing a major transition period before and after stolon development respectively. However, further insights into its tissue specific biological roles, to elucidate underlying regulatory functions in plant

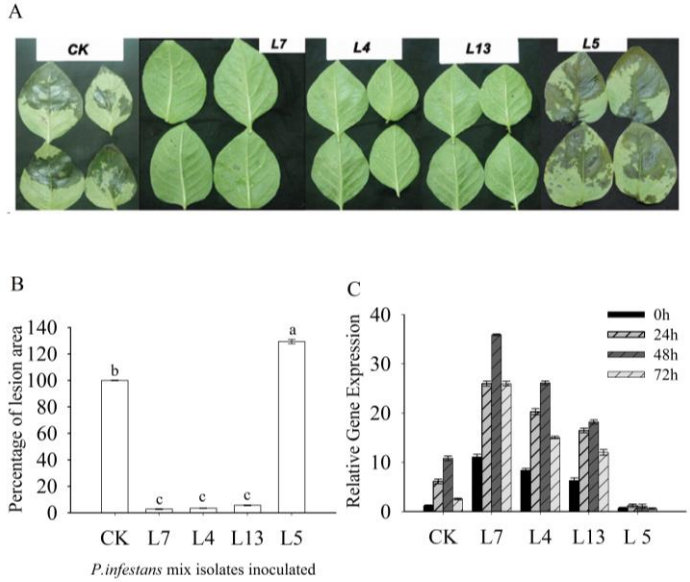

Fig 6. (A) Photographs of transgenic and control plants were taken six days after inoculation with *Pi* (B) Post inoculation disease lesion area was measured in control, overexpression and co-suppressed transgenic lines. (C) Relative expressions were measured for control and transgenic lines at different time points during inoculation process. Vertical bars bearing different letters indicate significant differences at $p < 0.05$.

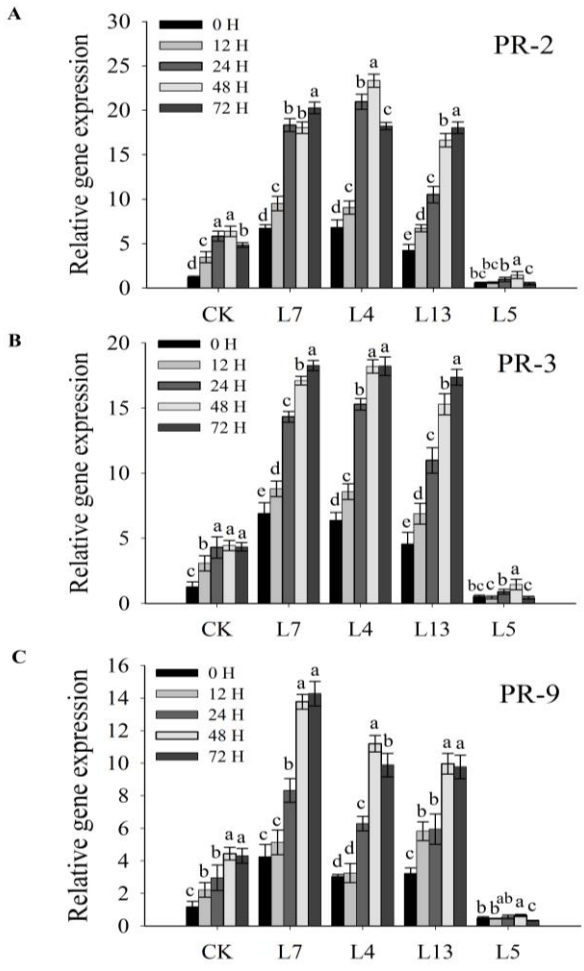

Fig 7. A comprehensive expression analysis of three important PR genes (A) PR-2 (B) PR-3 (C) and PR-9 were observed in details during inoculation for transgenic lines and untransformed control plants.Vertical bars bearing different letters indicate significant differences at $p < 0.05$.

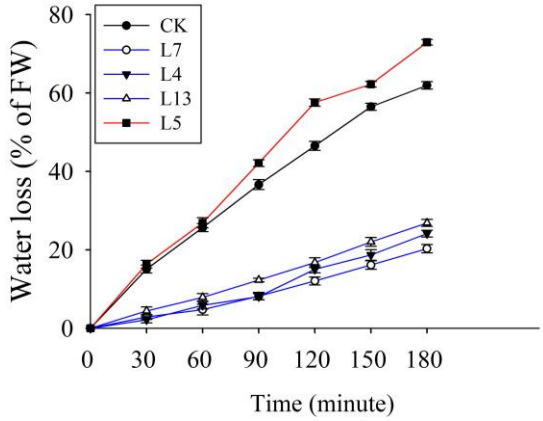

Fig 8. Detached-leaf water loss assay for transgenic and control (untransformed) plants, was performed and samples were taken at designated time points. Data for water loss was measured and represented as percentage of the fresh weight loss. Data is based on three independent replicates of six leaves from each line. Error bars indicate ± SE of means at $p < 0.05$.

development are still required. It is widely acknowledged that the plant hormones including SA, JA, ET and ABA play decisive roles in the signaling networks that tightly regulate the induced defense responses in plants (Van Wees et al., 2000; Von Dahl et al., 2007; Chen et al., 2013). Although synergistic and antagonistic responses were observed among plant hormones in different studies, but there is a growing evidence of simultaneous action of these signaling molecules to fine tune the optimal strategies including disease resistance, plant growth and development (Tang et al., 2013; Rajendra et al., 2009; Schenk et al., 2000). The WRKY factors induced by different plant hormones in various studies showing their involvement in basal defense networks. For instance, In *Arabidopsis*, 49 out of 72 WRKY genes were differentially regulated after infection by SA treatment (Dong et al., 2003). Moreover, In canola (*Brassica napus* L.), transcript abundance of 13 *BnWRKY* genes changed in response to one or more hormones, including ABA, 6-BA, JA, SA and ET (Yang et al., 2009). The *StWRKY1* expression markedly induced by action of SA, an important plant signaling hormone required for broad spectrum systemic acquired resistance (SAR). However, JA, ET and ABA also induced its expression, required for another layer of resistance independent of SA. It is impossible to understand SAR fully without knowing its interaction with other biological processes. It is hypothesized that plant defense pathways interact synergistically or antagonistically to fine-tune responses according to the challenging organism(s). The existence of SA-independent induced systemic resistance (ISR) pathway has been studied in *Arabidopsis thaliana*, which is dependent on jasmonic acid (JA) and ethylene signaling (Thomma et al., 2001). Moreover, simultaneous activation of different defense pathways by signal molecules has an additive effect on the level of induced resistance against pathogens (Van Wees et al., 2000). Similar results were implicated for a cotton gene (*GhWRKY3*), greatly up regulated after treatment with plant hormones and was involved in diverse stress responses (Guo et al., 2010). Based on our results, we conclude that *StWRKY1* play notable role between interaction of signaling pathways and its regulation is associated with multiple stress responses. Our first goal was to generate transgenic lines overexpressing the *StWRKY1* and evaluate their response after inoculation with *Pi*. We confirmed transgenic lines and analyzed their expression level to select for further experimentation, including co-suppression line (Fig.5). Co-suppression is not a rare phenomenon in genetically manipulated plants, to modulate the endogenous expression level of the gene and is common for the genes mainly involved in basal defense systems (Jorgensen, 1995; Ishikawa et al., 2005). The phenomenon of co-suppression usually occurs when multiple copies of particular sequences are present in the genome (Wang et al., 2012). Transgenic lines overexpressing *StWRKY1* demonstrated remarkable resistance upon inoculation with *Pi*. Interestingly, all the tested lines showed enhanced resistance except co-suppression line which further strengthened the importance of *StWRKY1* transcript accumulation in relation to *Pi* resistance. Many studies suggested that WRKY plant TF's regulate number of PR genes expression in response to attack by pathogens and provide improved resistance in transgenic plants. A recent genome-wide study in *Populus* revealed that constitutive expression of *PtrWRKY89* is directly involved in controlling number of antifungal PR gene expressions. Thus, it elevated the resistance of transgenic plants to *Dothiorella gregaria* (Jiang et al., 2014b). Hence, we investigated three important PR genes regulated by *StWRKY1* to link underlying molecular mechanisms with plant resistance. Transgenic lines overexpressing *StWRKY1*, showed enhanced expression of PR-2, PR-3 and PR-9. Interestingly, down regulation of these genes were observed in co-suppression line. Pathogenesis-related (PR) proteins that act as downstream components of systemic acquired resistance (SAR) in plants have been used routinely to investigate defense status of plants (Nawrath et al., 2002). PR-proteins are considered targets of WRKY TF's due to the presence of functional W-box in their promoter. A study reported by Park et al. (2006) intended to demonstrate that WRKY factors regulate the expression of PR genes which modulate defense response to various pathogens. Moreover, transcriptional regulation of PR genes by WRKY members positively regulate plant resistance against pathogens via plant signaling molecules (Tang et al., 2013). These investigations clearly suggesting that *StWRKY1* modulate plant resistance against *Pi*, by altering the expression of PR genes via complex signal transduction pathways, controlled by phytohormones. To further understand whether the *StWRKY1* involved in response to abiotic stress, we conducted dehydration assay. As described, preliminary evaluation of OE *StWRKY1* lines, showed that rate of water loss was markedly lower than that of control plants. Similar results were observed for wheat TF's (*TaWRKY2* and *TaWRKY19)*, corroborate enhanced resistance of transgenic lines to water stress conditions (Niu

et al., 2012). Surprisingly, CSP line L5 exhibited higher rate of water loss more than control (untransformed) plants, which further strengthened the diverse role of *StWRKY1* in stress situations. Various studies revealed that plant WRKY factors actively respond to abiotic stresses and positively regulate resistance thereby altering the expression of important stress-responsive genes. Such as, activated expression of *WRKY57* confers drought tolerance in *Arabidopsis* and the resistance was co-related with changes in the expression of stress-responsive genes (RD29A, NCED3, and ABA3). Further, they demonstrated that *WRKY57* can directly bind the W-box of RD29A and NCED3 promoter sequences (Jiang et al., 2012). In general, this study revealed significant reduction in the rate of water loss in OE plants as compared to untransformed control and CSP transgenic line, showing the involvement of *StWRKY1* in abiotic stress responses. However, a comprehensive study is required thereby investigating downstream responsive genes, to strongly co-relate biological functions of *StWRKY1* with abiotic stress.

Materials and methods

Plant materials and phytohormones preparation

Potato (cv. E-potato 3, E3) was used to generate transgenic plants and it is used as control (CK; untransformed E3) in this study wherever it is used. Expression level of *StWRKY1* was checked in different organs (leaf, stem, flower and tuber). Young leaves were sampled before stolon development, while mature leaf samples were taken when plants were actively producing tubers. The four important phytohormones with the following adjusted concentrations as described in other studies (Guo et al., 2010), SA (2 mM), JA (100 µM), ET (100 µM) and ABA (100 µM), were prepared. The leaves were sprayed (4-6 weeks old plants) with phytohormones and samples were collected at specified time.

Molecular cloning and transformation

The full-length sequence of *StWRKY1* gene was amplified from the cultivated E3 plants, using the gene specific primers (Table 1), designed according to the deposited NCBI sequence (Accession no: EF411203, Protein id: ABN69038). The amplified product was cloned into the cloning vector pMD18–T and sequenced (Sangon Biotech (Shanghai), Co. Ltd) for positive clone identification. A plant binary vector pBI121 (Accession no: AF485783), was used for overexpression of *StWRKY1* under control of CaMV35S promoter. To gain insight into subcellular localization of the target gene, complete ORF of *StWRKY1* was fused in frame with GFP (CaMV35S:*StWRKY1*:GFP). We used complete ORF of *Ghd7*, a member of transcript family, which was fused in frame with GFP (CaMV35S:*Ghd7*:GFP) and used as positive control for signal detection. Finally all the constructs along with RFP plasmid were introduced into *Agrobacterium tumefaciens* strain LBA 4404 by electroporation. The plasmids were injected into tobacco leafs (*Nicotiana tabacum*). The signals for GFP and RFP were detected by a confocal microscope (Zeiss, LSM510, Germany).

Plant regeneration

Bacteria (*A. tumefaciens)* containing plasmid pBI121-*StWRKY1* was cultured in LB containing 50 mg L^{-1} kanamycin and 50 mg L^{-1} rifampicin at 28 ºC in a shaker until the optical density reached at 0.5 OD_{600}. Briefly, according to Huai-Jun et al. (2003), microtubers obtained from the E3 plantlets, grown for 12-20 weeks, submerged in 20 ml bacterial solution for 5-10 min in 9 cm diameter petri dishes. Microtuber discs were then transferred onto petri dishes containing different shoot regeneration medium, and the plants with well-developed roots were propagated for further experimental analysis.

RNA isolation and qRT-PCR analysis

Genomic DNA of kanamycin resistant transgenic plants, was extracted and used as a template for further confirmation through PCR using *NptII* and primer pair for *StWRKY1*. Total RNA was extracted using TRIzol reagent (Sangon, Biotech (Shanghai), Co. Ltd) and quality of the total RNA was checked by OD_{260}/OD_{280} nm ratio, followed by treatment with DNaseI (TAKARA), and then reverse transcribed using reverse transcriptase (RT, Bio-Rad), RRI and Oligo(dT) (TOYUBO, JAPAN) reagents. The expression analysis was carried out through qRT-PCR using gene specific primers (Table 1). The potato *ef1α* gene (Accession no: AB061263) was used as internal standard control in qRT-PCR as it is relatively stable in expression. The qRT-PCR reaction was performed on the qPCR instrument (MJ Research, Bio-Rad, CFX connect™ Real-Time System). The following PCR cycling program was used: initial denaturation at 95 ºC for 30 s, followed by 40 cycles of 95 ºC for 10 s, 56º C for 30 s and 72 ºC for 5 s. Relative quantification of gene expression levels were calculated using a comparative Ct method as described by Cikos et al. (2007).

Inoculum preparation and detached-leaf inductions

Two aggressive isolates of *Pi* (99183 and 16-2) were used and grown on rye agar medium supplemented with 2% (w/v) sucrose and incubated at 18 ºC in the dark for 1-2 weeks. For inoculation, both isolates were collected and mixed and concentration was adjusted to 5×104 zoospores mL^{-1} to start the process of efficient inoculation for detached-leaf assay. Briefly, detached leaves (transgenic and control plants) were placed on water soaked papers, to meet the wet conditions necessary for inoculation, in the experimental trays maintained at climatic conditions of 16 h/8 h day/night photoperiod at 20 ºC. Leaves were spot inoculated (2 µl) on the abaxial side near the midrib of each leaf. Leaf samples were taken in the sampling tubes at defined time intervals and immediately dipped into liquid nitrogen and were kept at -70 ºC to perform qRT-PCR analysis. For disease assessment, the length (in mm) and width (in mm) of each lesion were measured at time points of 3, 4, and 6 days after inoculation. The lesion growth area were fully observed during the course of time and calculated (in mm^2) to judge the resistance level as described by (Vleeshouwers et al., 1999). Leaves were finally photographed at six DPI and data were presented in percentage of lesion area (where CK was scored as 100 %). Experiment was performed in triplicate with six leaves per inoculation.

Water loss assay

To inquire about the possibilities of potential role of potato *StWRKY1* gene in response to drought tolerance (abiotic stress), dehydration assay was performed as described in other studies (Ji et al., 2013). Three independent experiments were conducted and leaf samples were detached and immediately weighed (fresh weight in g, FW). Leaves were placed in petri dishes and shifted to laboratory bench (humidity, 40-50%, 22-24ºC) and weighed at specified

intervals (desiccated wight in g). Leaf samples were finally oven dried for 24 h to a constant dry weight (DW). Water contents (WC) were calculated using Formula:

$$WC(\%) = (\text{desiccated weight} - DW)/(FW - DW) \times 100$$

.

Bioinformatics and statistical analysis

The conserved domain of potato *StWRKY1* gene was clarified using the online NCBI tool for domain analysis (http://www.ncbi.nlm.nih.gov/Structure/cdd) to identify the putative functions of the WRKY protein. All experiments were performed in three independent replicates and the data were presented as mean values with standard deviations (SD). One-way analysis of variance (ANOVA) was carried out by using SPSS 16.0 software (SPSS, Inc., Chicago, III, USA). Differences were considered to be significant if $P < 0.05$.

Conclusion

In conclusion, constitutive expression of *StWRKY1* enhanced the resistance of transgenic potato plants against late blight disease. OE lines exhibit resistance thereby altering the expression of PR genes activated by a network of defensive pathways. Involvement of *StWRKY1* in broad spectrum signal transduction was confirmed by its up regulation, observed under treatment of important plant signal molecules. Furthermore, It is speculated that *StWRKY1* is also important to modulate plant resistance to abiotic stresses demonstrated by preliminary dehydration test. Due to the fact that *StWRKY1* gene responds to phytohormones, fungal pathogen, dehydration stress and accumulated in different organs, as a transcription factor, *StWRKY1* might be one of the key regulatory genes involved in plant defense responses and potato development. However, to further investigate its physical interaction with downstream stress responsive and developmental related genes, a detailed research is required at the protein level.

Disclosure statement

No competing financial interests exist.

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