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HETEROLOGOUS EXPRESSION OF OsRGLP1 GENE INDUCES RESISTANCE AGAINST FUSARIUM INFECTION IN TRANSGENIC POTATO

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ABSTRACT

Germins and germin-like proteins (GLPs) are a large and extremely diverse family of plant proteins that are present ubiquitously and are also involved in many of the processesimportant for plant development and defense mechanism. Potato is susceptible to many kinds of diseases, especially to fungal pathogens, therefore genetic engineering of potato for disease resistance is an important strategy study and apply disease resistance. GM technology can be an effective tool for crop improvement. The transgenic approach was pursued to introduce rice (*Oryza sativa*) germin like protein gene *OsRGLP1*via *Agrobacterium* mediated transformation.Confirmation of the presence of the gene was carried out by polymerase chain reaction (PCR). One-step reverse transcriptase PCR was used for transcriptional analysis and expression was quantified in real time in all PCR positive putative transgenic lines. Three high expression lines were selected to check the effect of transgene *OsRGLP1* on morphology and growth. A significant difference was observed in plant height, shoot number, number of leaves and tubers harvested in comparison with wild type control. Overexpression of *OsRGLP1* exhibited increased foliar resistance when exposed to *Fusarium oxysporum* f.sp. *tuberosi* in transgenic lines suggesting its role in nonspecific fungal resistance.The results from the present study suggest an important role of *OsRGLP1* in establishment of plant defense responses.

Keywords: *Solanum tuberosum,* Germin-like protein, *Fusarium oxysporum,* heterologous expression, genetic transformation.

INTRODUCTION

Germin like proteins are a huge and diverse family of plant proteins. Proteins from GLPs family are present in all organs and at all developmental stages and many of them are also involved in several stress related responses. It is thus obvious that these proteins may be involved in many of the processesimportant in plant development and defense mechanism (Bernier and Berna). Germins and germin-like proteins (GLPs) have been associated to solidify plant cell wall, thereby deliver resistance to environmental stresses in plants. There are numerous illustrations available linking GLP expression to plant defense and recommend GLPs as markers in the defense mechanism of the plant (Wei *et al*., 1998; Zimmermann *et al*., 2006; Godfrey *et al*., 2007). GLPs/SOD catalyzes the conversion of superoxide to H_2O_2 and O_2, which is of major importance in protecting living cells from toxicity produced by superoxide anion under oxidative stress conditions. Several SODs might be involved in controlling oxidative stress in many cell compartments such as chloroplast, peroxisomes and mitochondria (Alscher *et al*., 2002).León-Galván *et al*. (2011) proposed that the *Cch*GLP gene codes for a GLP with Mn-superoxide dismutase activity and suggested a probable role for *Cch*GLP in pathogen resistance, and likely with salicylic acid and ethylene signal pathways involved in these events. The down-regulation of *OsGLP1* in an indica rice cultivar by siRNA-mediated gene silencing showed significant reduction in its expression and exhibited decreased height and were extremely affected by fungal pathogens. Its increased expression in transgenic tobacco plant has recognized its relationship with cell wall and enzymatic activity as SOD also suggests its involvement in plant height regulation and disease

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resistance(Banerjee *et al*., 2010). The introduction and expression of defense related proteins into plant genomes have shown to inhibit phytopathogenic fungal invasion significantly. Disease control can never be accomplished absolutely, however the management of diseasedepends on the approach employed and characteristics of the fungal pathogen. The communication between the expressed gene product, plant species, and phytopathogen is a complex mechanism which designates response of transgenic plants to this stress. Introduction of more than one defense gene has shown extensively more potential in providing disease resistance than single transgene introgression (Punja, 2001).

Potato (*Solanum tuberosum*L.) is known to be a crop of agronomical importance and is ranked fourth worldwide in terms of demand and production (Chakravarty *et al.,* 2007). In Pakistan it is ranked third among food crops next to rice and wheat and fifth for its total production. It is an important vegetable crop due to its natural prospects for high production, cost-effective income and nutritious values (Bhutta, 2008). Potato crop faces loss in production due to many diseases caused by fungi, viruses. The area under potato cultivation is increasing, there is lack of information regarding disease management, disease free hybrid seed is not widely available, therefore, the diseases are becoming complex and taking lion's share in potato (Bhutta and Hussain, 2002; Younis *et al*., 2005).

Development of disease resistance is one of the several classical approaches to disease control. *Agrobacterium* mediated transformation is nevertheless the main technology used for the production of genetically modified plants (Beaujean *et al.,* 1998).The genetically modified (GM) varieties of main agronomic crops, especially rape (canola), cotton, soybean and maize were among the first grown commercially in 1996. General impression of GM crops in the developed and developing countries has been positive. Generally, GM crops have evidenced to be an optimistic addition to most of the technologies which consist of up-to-date agriculture (Mannion and Morse, 2013).

The objectives of the present study were to generate potato lines with stable integration of transgene*OsRGLP1* and to determine the antifungal activity of this gene in transgenic potato lines against *Fusarium oxysporum* f.sp. t*uberosi*.

MATERIALS AND METHODS

Plant material: The potato variety *Desiree* was clonally maintained *in vitro* using MS medium at 25±3°C in incubator in an intensity of approximately 2000 lux with a photoperiod of 16hrs.

Fungal strain: Pure cultures of *Fusarium oxysporum* f.sp. *tuberosi*wereobtained from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences (IAGS), Punjab University Lahore andwere maintained on potato dextrose agar (PDA) at 27±5°C for fungal assay.

Potato transformation and PCR analysis: The recombinant vector pC:*Os*RGLP1 containing *OsRGLP1* gene under the transcriptional control of the 35S promoter (Figure 1) was electroporated into *Agrobacterium tumefaciens* strain GV3101. Transgenic lines were generated by using a protocol developed at potato breeding and genetics lab Michigan state university, USA. The internode cuttings of stems were taken as explants from tissue culture plantlets. Shoots of 1 to 1.5 cm length were carefully excised and transferred to rooting medium (MS medium+ 15 mg/L hygromycin) in test tubes and were placed at 25±3°C in an incubator in a light intensity of approximately 2000 lux with a photoperiod of 16 hrs. To make sure that regenerated plants were obtained from independent transformation events, a single shoot was removed from each callus. Roots were developed after 2 to 3 weeks. Hygromycin selected rooted transformants were maintained by micropropagation (Figure 2).

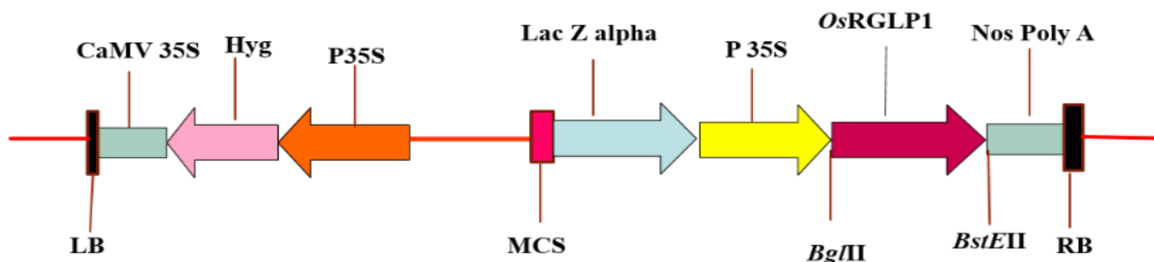

Figure 1. T-DNA region of pC-*Os*RGLP1 specifies *OsRGLP1* gene (brown arrow) under the transcriptional control of CaMV35S promoter. Construct contains hygromycin gene (pink arrow) for plant selection and kanamycin gene (not shown) for bacterial selection outside the T-DNA region.

Figure 2. Different stages of potato transformation and regeneration A: Two week old explants on selection medium B: Shoots generating from four week old explant on selection medium C: complete transformed plant on MS medium without growth hormones D: Soil acclimatized transgenic plant E: Minitubers from transgenic plants.

The total genomic DNA was isolated by using DNeasy plant mini Kit (QIAGEN Valencia, California, USA). The *OsRGLP1*gene transfer was confirmed through PCR usingspecific pair of primers, forward primer for 35S (CaMV) promoter and reverse primer specific for gene (Table 1). The thermal profile adopted for PCR was 94°C for 3 min followed by 35 cycles each of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and final extension at 72°C for 10 min.

Table 1. Primers used in the molecular analysis experiments.

Primer Name	Primer sequence 5' to 3'	Product size (bp)	Accession No.
CaMV P-F	5'-CTATCCTTCGCAAGACCCTTC-3'	958	AFAF234297.1
RGLP1-R	*BstE*II5'-CTCGAGGTGACC GTCACAAAGAACACTG-3'		AF141878
RGLP1-F	*Bgl*II5'-ATCTAGATCT CATCTCAAACACACCACC-3'	958	AFAF234297.1
RGLP1-R	*BstE*II5'-CTCGAGGTGACC GTCACAAAGAACACTG-3'		AFAF234297.1
EF-1α-F	5'-GGTGGTTTTGAAGCTGGTATCTC-3'	400	AB061263
EF-1α-R	5'-CCAGTAGGGCCAAAGGTCACA-3'		AB061263
RTGLP-F	5'-CACTCCTCGGAAGACGAAC-3'	265	AFAF234297.1
RTGLP-R	5'-CCCACAGGGAATACGAACAC-3'		AFAF234297.1
EF-1α-F	5'-AGAAGGTCGGTTACAACCCTGA-3'	281	AB061263
	5'-TACCACCAGTAGGGCCAAAG-3'		AB061263

Transcript Analysis: To analyze the *OsRGLP1* expression, total RNA was isolated from transgenic as well as wild type control plants by using RNeasy Plant Mini Kit (QAIGEN Valencia, CA). TURBO DNA-free™ Kit was used to remove DNA contamination from RNA preparation. One step RT-PCR was conducted using the platinum *taq* system (Invitrogen by life technologies) and the reaction was prepared according to the manufacturer's guidelines. The thermal profile included cDNA synthesis and pre-denaturation, PCR amplification and final extension. The first step included 1 cycle of 45°C for 30 min followed by pre denaturation for 2 min

on 94°C. The second step included 35 cycles of: denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 40 s. Third step included 1 cycle of 72°C for 10 min.Primer pairs used are listed in table 1. Amplified products were separated using 1% agarose gel.

qRT-PCR analysis: Total RNA was isolated from plant tissues using Thermo Scientific GeneJET Plant RNA Purification Mini Kit according to manufacturer's instructions.The extracted total RNA was used for cDNA synthesis using RevetAid First Strand cDNA Synthesis Kit (Thermo Scientific cat #K1621).Before quantifying the expression, the cDNAs were subjected to routine PCR using primers for *EF-1α* (table 1) and the result was seen on 1% agarose gel. Relative quantification of expression of *OsRGLP1* in independent transgenic lines was performed by using Maxima SYBR Green qPCR Master Mix (2X) kit (Fermentas Life Sciences Cat# K0221).The reaction conditions for real time PCR included one cycle of pre-amplification denaturation at 95°C for ten min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 30 s and extension at 72°C for 30 s. The relative quantification was done with EF-1α as a reference gene using Line-Gene K Fluorescence Quantitative PCR Detection System (BIOER).The primer pairs used in real time PCR are listed in the table 1. The method used by software for relative quantification was $2^{-[delta]\,[delta]}$ Ct method.

Morphology and Growth Analysis: Morphology of 3 months old plants of wild type control and three selected high expression lines was observed. Twenty four plants were grown in greenhouse, 6 for each type and data was collected for plant height, number of shoots, number of leaves and tubers harvested per plant.

Disease Incidence Assay: Fungal resistance ofun-transformed and transformed potato plantswastested against *Fusarium oxysporum* f.sp. *tuberosi.* Fungal inoculum was prepared using sorghum seeds according tothe method used by (Akhtar *et al.,* 2005). The sterilized clay and sand mixture was mixed with a weighed inoculum of *F. oxysporum* f.sp. *tuberosi* and incubated for one week at 25°C. The pots were filled with infected clay and sand mixture. Six weeks old potato plants from greenhouse both wild type control (D-wt) as well as high expression lines(DC-1, DC-2, DC-7) were shifted to the pots and watered as required. Plants in pots without inoculum served as negative control. Pots were kept in greenhouse at 25°C under natural light. Symptoms were scored at 12, 15, 18 and 21 days after planting based on modified scale of Silva and Bettiol (2005).

Statistical Analysis: Data was scored and analysis of variance (ANOVA) and DMRT was calculatedusing software MSTATC.

RESULTS

Transformation efficiency calculation and selection of transcript positive lines: A total of 200 internodes were co-cultured with *agrobacterium* strain GV3101, out of which 114 explants were regenerated on selection medium containing 15mg/L hygromycin (Table 2). Hygromycin selected plants were confirmed by PCR amplification of the 958 bp fragment of the *OsRGLP1* gene (Figure 3). Among regenerated rooted shoots, 35 plantlets were selected for DNA isolation and detected for presence of gene of interest by PCR. Transformation efficiency was 75% calculated according to Jo *et al*., 2014 which also counts shoot regeneration efficiency and is defined as the percentage of PCR positive rooted shoots (Table 2).

Table 2. Transformation of *OsRGLP1* gene to Potato *cv Desiree.*

Vector Parent/ recombinant	Explant No.	Regeneration time (days)	Hygromycin selected explant #	Regeneration%	Shoot %	Shoot No.	Rooting %	PCR⁺ %	Transformation frequency %	Transgenic lines selected No.	RT-PCR⁺ lines %
pCAMBIA1301/ pC:*Os*RGLP1	200	60-120	114	100	71	35	100	75	53	9	77

Shoot %; percentage of number of shoots over number of explants, rooting %; percentage of number of rooted shoots over number of shoots, PCR⁺ %; percentage of PCR positive shoots over the number of shoots, transformation frequency %; calculated as shoot % × rooting % × PCR⁺ %. Among all regenerated shoots, 35 plants from pC:*Os*RGLP1 were tested by PCR for gene presence. RT-PCR⁺ lines %; percentage of transcript positive lines over selected PCR⁺ lines tested through one step reverse transcriptase PCR.

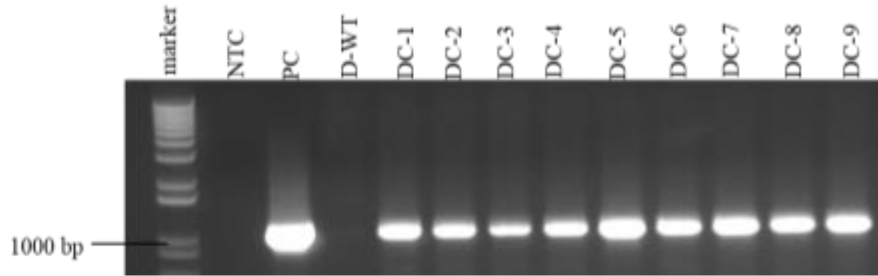

Figure 3. PCR amplification of transgene OsRGLP1. The expected *OsRGLP1* fragment is 958bp, lane NTC, no template control, lane PC, plasmid pC:*Os*RGLP1 positive control, lane D-WT, internal control, lanes DC-1 to DC-9, independent transgenic lines. 100bp DNA ladder was used as a molecular weight marker.

Transformation frequency calculated was 53% when defined as the percentage of PCR positive events among the number of explants used for transformation.For detection of expression of *OsRGLP1* in potato one step reverse transcriptase PCR was performed using platinum *taq* as a substitute approach to estimate the copy number. Amplification of all selected independent transgenic lines showed bands of 400bp with primers used for housekeeping gene *EF-1α* (Figure 4A). The amplification with gene specific primers showed interesting results. Out of nine PCR positive, independent lines 7 lines were *OsRGLP1* transcript positive detected as 958bp band while two lines showed no expression (Figure 4B). Sets of primer pairs used are listed in Table 1.

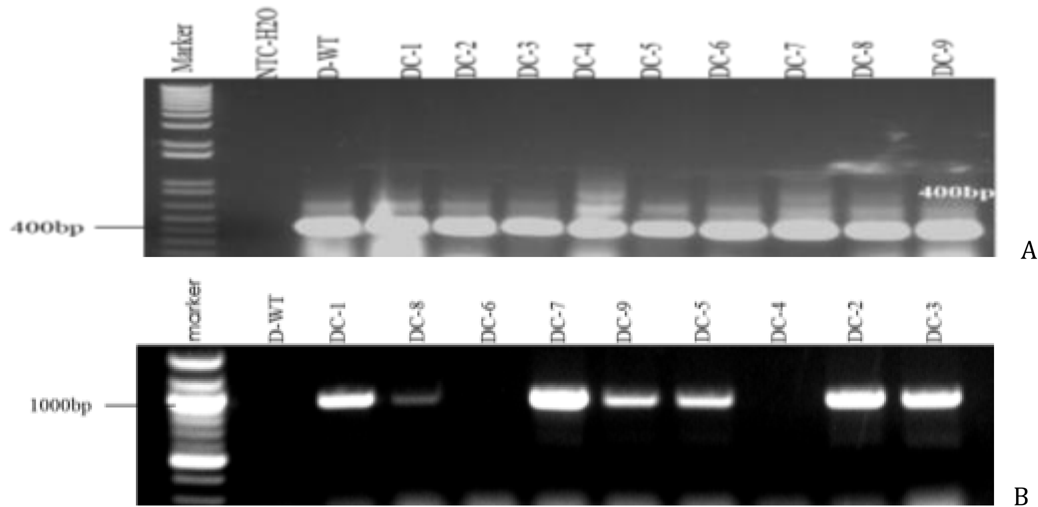

Figure 4. One step Reverse Transcribed PCR of *EF-1α* and *OsRGLP1*. All the transgenic lines as well as untransformed wild type (D-WT) were transcript positive with EF-1α primers depicting a band of 400bp (A). In PCR with gene specific primers D-wt served as internal control showing no expression. Out of nine lines PCR+ lines seven were transcript positive (958bp band) while DC-4 and DC-6 were negative (B).

Real time PCR analysis: Relative gene expression analysis was conducted by using qRT-PCR technique. *EF-1α*served as reference gene to normalize the expression levels of *OsRGLP1*.The cDNA from all PCR positive lines and wild type control plant when subjected to routine PCR with primers for *EF-1α* showed amplicons of 281bp (Figure 5A). Five out of 7 were observed as high expression lines. Two lines (DC-4 and DC-6) showed negligible expression with line DC-8 exhibiting lower expression (Figure 5B). These results coincide with the results from one step RT-PCR (Figure 4B).

Comparative Analysis of Morphological characteristics: Three selected high expression transgenic lines and wild type control plants were grown in greenhouse condition to study the effect of *OsRGLP1* gene on different morphological characteristic. The parameters included plant height, number of leaves, shoots and tubers harvested per plant. Observations were made on 3 months old plants of wild type control and high expression lines, 9 plants for each type. All high expression lines revealed significant differences in plant height, number of shoots, number of leaves and number of tubers as compared to wild type plants (Figure 6A&B).

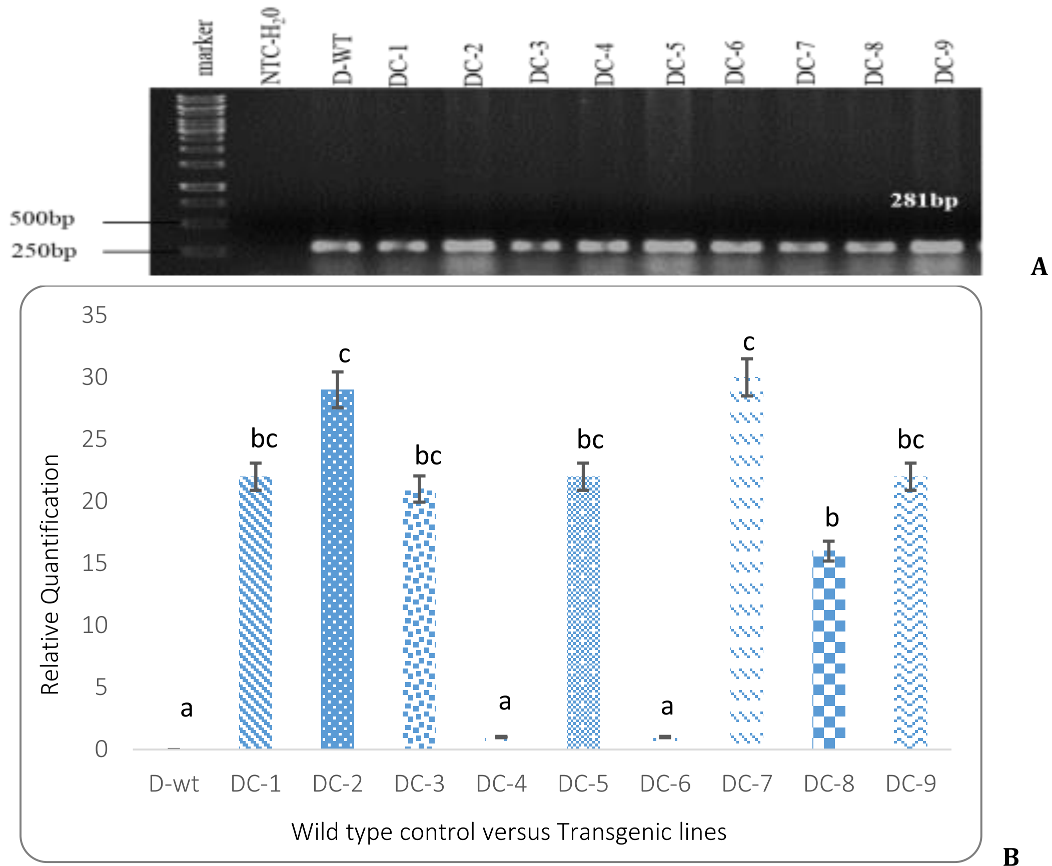

Figure 5. A) Reverse transcriptase PCR with EF-1α. The expected fragment of EF-1α is 281bp, NTC-H2O, no template control, D-WT, untransformed internal control. B) Real time quantitation of *OsRGLP1*. Transcript level for *OsRGLP1* gene in control and transgenic lines was normalized with the EF-1α messenger RNA level. D-wt, untransformed wild type served as control. Transcript levels were significantly higher in transgenic lines than in the wild-type control. Significance determined with the DMRT ($p < 0.05$) is indicated by letters.

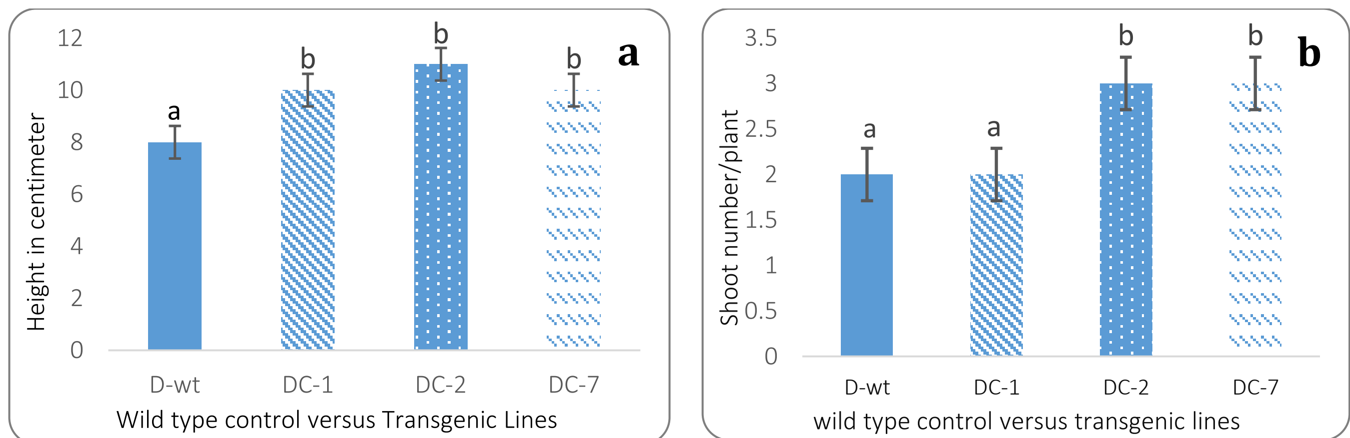

Figure 6A. Morphology and growth analysis: a) Comparison of plant height of 3 month old wild type plant and 3 independent transgenic lines. B) Comparison of number of shoots. Data is average 9 plants for each line as well as wild type. Significance was tested with DMRT ($P \leq 0.05$).

Anti-fungal Assays with Selected High Expression Lines: To evaluate the effectiveness of *OsRGLP1* in creating resistance against pathogenic fungi the high expression lines and non-transformed potato plants were infected with *F. Oxysporum* f.sp. *tuberosi.* Disease scoring was categorized into the six classes from 1 to 6,

based on the expansion of the symptoms (Table 3). Clear differences were noticed in the development of symptoms of Fusarium wilt between transgenic lines and wild type control plants (Table 3). Twelve days after infection, disease symptoms were seen on wild-type plants (D-wt), in the form of yellowing of leaves, mainly on older leaves. Transgenic leaves showed no symptoms after 12 days, slight yellowing of leaves was seen 21 days after infection (Table3).

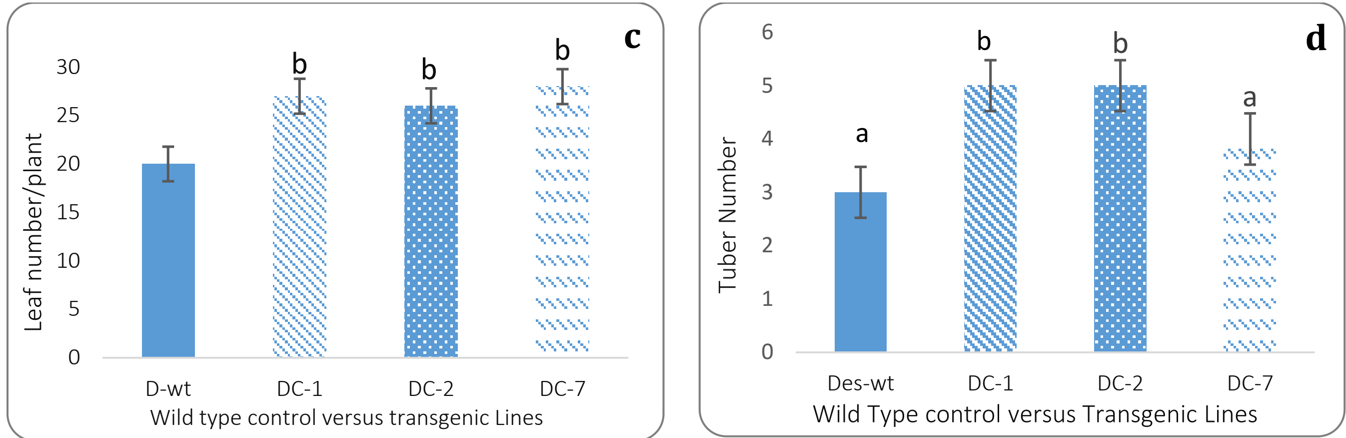

Figure 6B. Morphology and growth analysis **c)** Comparison of number of leaves. **d)** Comparison of tubers harvested per plant. Data is average of 9 plants for each line as well as wild type. Significance was tested with DMRT ($P \leq 0.05$).

Table 3. Fusarium wilt incidence scoring on potato plants infected with fusarium *oxysporum* f.sp. *tuberosi.*

		Disease scores at days post inoculation			
Line	No. of Plantlets	12	15	18	21
D-wt uninfected	18	1	1	1	1
D-wt Infected	18	3	4	4 to 5	5 to 6
DC-1 uninfected	18	1	1	1	1
DC-1 Infected	18	1	1	1 to 2	2
DC-2 uninfected	18	1	1	1	1
DC-2 Infected	18	1	1	1 to 2	2
DC-7 uninfected	18	1	1	1	1
DC-7 Infected	18	1	1 to 2	2	2

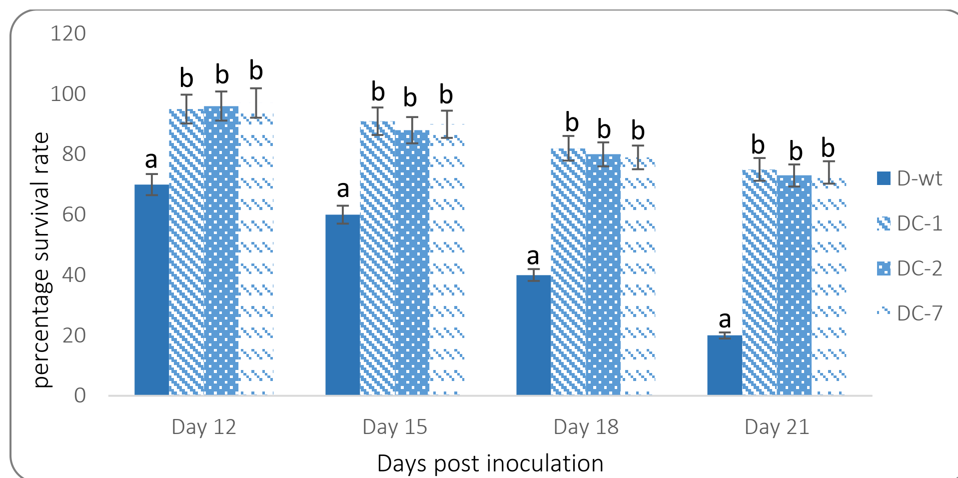

Figure 7. Survival rate of potato plants infected with *fusarium oxysporum* f.sp. *tuberosi.* Plants of three high expression lines (bars with different patterns) and wild-type D-wt (blue) were infected with *F. oxysporum* f.sp. *tuberosi*. Six week old potato plantlets were shifted to infection mixture (sand + clay + inoculum) and the survival rate in % was calculated 12 and 15, 18 and 21 days post-inoculation. Bars indicate standard errors, based on three independent experiments. The

data represents significant differences in survival rates for transgenic lines and the wild-type control plants based on DMRT ($p \leq 0.05$), as indicated by letters.

After 21 days wild type plants suffered seriously from fungal infection, and more than 70% of the leaves displayed severe infection symptoms (score 5 to 6) while for transgenic plants very minor infection symptoms were noticeable on the oldest leaves represented by scoring 1 to 2 (Table 3).

The Survival rate percentage was calculated 12, 15, 18 and 21 days post inoculation. A significant difference was observed between transgenic lines and wild type plants when data were analyzed using DMRT (Figure 7). These results advocate that the over-expression of *OsRGLP1* gene in potato decreases the susceptibility of the potato plants to *Fusarium oxysporum* f.sp. *tuberosi*.

Plants of three high *OsRGLP1* expression lines DC-1, DC-2, DC-7 and wild-type control D-wt were infected with *Fusarium oxysporum* f.sp*. tuberosi.* Disease symptoms scoring is based on modified scale of Silva and Bettiol (2005): 1, no symptoms; 2, plant showing yellowing of leaves and wilting (1 to 20 %) ; 3, plant showing yellowing of leaves and wilting (21 to 40 %); 4, plant showing yellowing of leaves and wilting (41 to 60 %); 5, plant showing yellowing of leaves and wilting (61 to 80 %); 6, plant showing yellowing of leaves and wilting (80 to 100 %) or die. Three independent experiments were carried out. Plants without infection considered as control.

DISCUSSION

GLPs have been proposed to exhibit a crucial role in numerous characteristics development and potential involvement in plant basal host resistance. The mechanism by which GLPs influence plant defense is related to their generation of higher levels of H_2O_2 and active oxygen species. The Chromosome 8 QTL which is associated with *OsGLP* genes when introgressed into rice can add to disease resistance against phytopathogenic fungi in the field (Manosalva *et al*., 2009). León-Galván *et al*., 2011 studied *CchGLP* gene and suggested that their results support a possible role of this gene in pathogen resistance. Germin and GLPs are said to be involved in defense against a broad range of pathogenic fungi (Zimmermann *et al.,* 2006). Christensen *et al*. (2004) described that the transient overexpression of *Ta*GLP4 and *Hv*GLP4, may contribute in providing resistance to *Blumeria graminis* in wheat and barley. A germin like protein *Bv*GLP1from sugar beet (*Beta vulgaris)* when introduced into *Arabidopsis thaliana* showed enhanced resistance to soil born phytopathogen *Rhizoctonia solani* (Knecht *et al.,*2010). The introduction and expression of defense related proteins into plant genomes have shown that the progress of phytopathogenic fungi can be reduced significantly. Development of disease resistance is one of the several classical approaches to disease control. The *Agrobacterium* mediated transformation was done through *Agrobacterium* strain GV3101 harboring *OsRGLP1* gene using internode/stem pieces as explants. *Agrobacterium*-mediated genetic transformation has long been used and a preferred method to generate transgenic plants. *Agrobacterium tumefaciens* is the only cellular organism which can infect a broad range of plant species mostly dicots (De Cleene and De Ley, 1976) and some monocots (De Cleene, 1985). Potato being a dicot plant is a natural host to this bacterium. A total of nine PCR confirmed independent transgenic lines were obtained and were subjected to one step reverse transcriptase PCR for expression analysis. To confirm the presence of RNA and to normalize the expression in different samples primers for *EF-1α* were used in PCR. *EF-1α* (AB061263) has previously been used as internal control by different researchers (Nicot *et al*., 2005, Chen *et al*., 2010, Kuhl *et al*., 2007). The amplification with gene specific primers showed interesting results. Out of nine PCR positive, independent lines 7 lines were *OsRGLP1* transcript positive while two showed no expression (Figure 4B). The absence of *OsRGLP1* transcript in two PCR positive lines could be described by a fractional deletion/rearrangement of the *OsRGLP1* transgene, leaving intact the 958bp DNA region, but inactivating or truncating transcription (Kuhl *et al*., 2007). Felcher *et al*., 2003 detected post-transcriptional silencing in other transgenic lines, this also explains the transcript absence. Real Time-PCR is considered as a very sensitive method for the detection and relative quantification of low abundance mRNAs (Bustin, 2000), and can be used for the analysis of expression of gene in specific tissue (Bustin *et al*., 2000), and for plant studies (Gachon *et al*., 2004). To analyze gene expression precise, sensitive and reproducible measurements are essential for specific mRNA sequences. Therefore the choice of proper internal control is very essential to normalize the expression level. According to Nicot *et al*., 2005 the expression of EF-1α did not seem to be influenced during salt, cold and late blight stresses, in the present

study it was selected to use as a reference gene to normalize the expression levels of *OsRGLP1*. Five out of seven transgenic lines were observed as high expression lines, two lines showed negligible expression with line DC-8 exhibiting lowest expression (Figure 5B). These results coincide with the results from one step RT-PCR (Figure 4B). GLPs may play regulatory role in different developmental stages of plants such as leaf, root, flower, seed and fruit development (Dunwell *et al*., 2008). Constitutive expression of *OsRGLP1* in potato could affect its morphology. Three high expression lines were selected to study the effect of gene (*OsRGLP1*) on morphology and growth. The parameters included plant height, number of leaves shoots and tubers harvested per plant. All high expression lines showed significant difference in plant height as compared to wild type control plants (Figure 6A, **a**). Vector backbone integration did not play any role, this increase in plant height in transgenic lines could be due to *OsRGLP1* expression, as GLPs are identified as proteins which are involved in developmental regulation of plants. It has already been suggested that the generation of $OH^-$from H_2O_2 may directly play a role in cell wall slackening by polysaccharide cleavage (Fryer*et al*., 2002; Liszkay *et al*., 2004). In the present study, the increased height obtained in transgenic plants could be due to the activity of transgene *OsRGLP1* that resulted in the generation of high H_2O_2 which may be one of the reasons of expansion in the cell wall. Banerjee and Maiti (2010) exposed the efficient role of rice *Os*GLP1 in plant height regulation by establishing transgenic rice plants through gene silencing. Number of shoots when counted per plant significant difference was observed in two out of three transgenic lines when compared with wild type control (Figure 6A, **b**). A number of properties/functions have been associated with GLPs, one of which is their participation in the regulation of different stages of plant development. (Dunwell *et al*., 2008). Increase in leaf number can be correlated to the role of GLPs in plant height as one report has shown that *Os*GLP1 regulate plant height development (Banerjee and Maiti 2010). In the present study, we observed an increase in leaves number per each plant as compared to wild type control (Figure 6B, **c**). The increase in leaf number of transgenic plants can be attributed to the involvement and over expression of *OsRGLP1* gene. Germin like proteins are most ubiquitous plant proteins and their expression is linked to number of traits, they are differentially expressed during particular periods of plant growth and development. Over expression of germin like protein gene in transgenic plant may play a role in the better development of morphological characteristics. Numbers of tubers harvested for each plant were counted from both wild type control and transgenic lines for each replicate. Significant increase in tuber number was observed for transgenic lines compared to wild type control (Figure 6B, **d**). The high production of H_2O_2 and superoxide radical ($\bullet O_2^-$) is a common characteristic that plant displays during defense responses generated by the plant whenever challenged to elicitors and microbial pathogens (Lamb and Dixon, 1997). It has been suggested that a fast increase in both extra and intra cellular H_2O_2 is involved in the initiation and/or implementation of the hyper sensitive response (Levine *et al*., 1994; Bestwick *et al*., 1998). There is significant evidence that H_2O_2 has an extensive role in resistance responses, because it is essential for crosslinking of cell wall constituents as part of structural defense reactions and may also control gene expression connected with phytoalexin biosynthesis, antioxidant defenses and improvement of systemic acquired resistance (Lamb and Dixon, 1997). To assess the role of *Os*RGLP1 in pathogenic fungi interaction the high expression selected transformed lines and non-transformed potato plants were infected with *F. Oxysporum* f.sp*. tuberosi*. The infected plants were observed daily and were scored after every four days and categorized into the six classes from 1 to 6, established on the expansion of the disease symptoms (Table 3). Strong differences were detected in the progression of the disease symptoms between transgenic and wild type plants. The survival rate percentage was calculated 12, 15, 18 and 21 days post inoculation. A significant difference was observed between transgenic lines and wild type control plants when data were analyzed using DMRT test (Figure 7). These findings advocate that the over expression of *OsRGLP1* in potato lessens the susceptibility of the potato plants to *Fusarium oxysporum* f.sp.*tuberosi.*Zimmermann *et al*., 2006 improved the tolerance in barley by transient overexpression of *Hv*GER5 gene in epidermal cells and detected that added resistance is reliant on its SOD activity. A promising description of the antifungal function of *Os*RGLP1 in potato plant could be the production of H_2O_2 due to its enzymatic activity (SOD or OxO). H_2O_2 helps in the papillae formation by crosslinking cell wall proteins

at the infection sites. This cell wall strengthening helps the cell to protect itself against fungal penetration (Wei *et al*., 1998; and Christensen *et al*., 2004). H_2O_2 produced by *Os*RGLP1 may also function as a signaling molecule by triggering other defense responses in plants so *Os*RGLP1 may be employed for general defense against fungal infections. The high expression transgenic potato plants were not completely protected against infection by *F. oxysporum* f.sp. *tuberosi* however, the delay in disease progression was observed, over-expression of this gene may provide a general defense system against fungi at least in potato. Our findings demonstrate that *OsRGLP1* could be a favorable candidate gene for crop improvement through genetic engineering strategy.

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