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Identification and expression analysis of *TLPs* as candidate genes promoting the responses to both biotic and abiotic stresses in wheat

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Abstract

Thaumatin like protein (*TLPs*) gene family have the ability to respond to both biotic and abiotic stresses. In this research, gene expression and bioinformatics analysis were applied to reveal the function of *TLPs* more evidently. Real Time PCR technique was used to compare *TLP* gene expression between susceptible and resistant cultivars of wheat under the *Mycosphaerella graminicola* inoculation. Our results indicated the early resistant-related response by *TLP* up-regulation within 3h after inoculation. In abiotic stress we observed induction of *TLP* gene expression during salt stress in susceptible cultivar. 50mM NaCl induced rapid induction of *TLP* within 3h, while 100mM NaCl conferred *TLP* up-regulation later at 72h. *In silico* chromosome walking analysis revealed that four loci can be the candidates for conferring tolerance to both biotic and abiotic stresses. In this study, a combination of Real Time PCR technique and bioinformatics tools suggested the possible role of *TLP* homologs in response to both biotic and abiotic stresses.

Keywords: Biotic and abiotic Stresses; Gene expression; *In silico* chromosome walking; Thaumatin like protein.
Abbreviations: ABA-Abscisic acid; ACC-1-aminocyclopropane-1-carboxylic acid; BLAST-Basic local alignment search; CT-Cycle threshold value; ECM-Extracellular matrix; EF-1α-Elongation factor 1-α; ET-Ethylene; FHB-Fusarium head blight; HR-Hyper sensitive response; INA-2; 6-dichloroisonicotinic acid; MJ-Methyl Jasmonate; ORF-Open reading frame; PR-Pathogenesis related protein; RT-PCR-Reverse transcription polymerase chain reaction; SA-Salicylic acid; TF-Transcription factor; TLP-Thaumatin like protein; TMV-Tobacco mosaic virus.

Introduction

Different kinds of stresses threaten crop plants, followed by decreasing in crop yield and quality. The ascomycete fungus *Mycosphaerella graminicola* (asexual stage: *Septoria tritici*), causes an economically important disease of wheat, *Septoria tritici* leaf blotch. Leaf blotch, caused by *Septoria tritici* is one of the main diseases of wheat in more than fifty countries (Eyal, 1987). The initial stages symptoms of the disease are the clear yellow, small, globular or oblong blotches. In the more advanced stages of infection, the blotches can cover all of the leaf and sheath and become linear and almost parallel to the veins (Gieco et al., 2004). The detailed studies of the interaction between *S. tritici* and host have only recently begun (Rudd et al., 2008). The use of fungicide against this pathogen is not economical and reliable; therefore, the most effective and environmentally safe approach to conflict this disease is genetic resistance (Eyal, 1981). Commonly, the ideal goal in genetic engineering is to produce resistant plants against wide range of stresses such as drought, salinity, fungal stress, etc. Regarding multigenic nature of stress tolerance, coordinate manipulation of different stress-pecific genes should perform to confer resistance to more than one stress in a plant. However, due to limitations in transformation of a lot of genes, it is unpractical to manipulate multiple genes coordinately. Consequently, to deal with stresses such as *Septoria tritici* leaf blotch which are induced by multiple genes (Rajam et al., 2007), one of the most significant current discussions is to discover the genes which their transformation can confer resistant to more than one stress.

Transcription factors (TFs) were the first gene family, whose transformation has the potential to switch on a number of resistant genes involve in a variety of biotic and abiotic stresses (Archana et al., 2009). The reason of that is thought to be the capability of TFs in activating of shared elements in different regulatory networks. For instance, more than 50% of expressed proteins in biotic and abiotic stresses are similar to WRKYs transcription factors in Arabidopsis (Kalde et al., 2003). However since various roles of TFs in different regulons are not completely clear, manipulating of TFs has the risk of inducing undesirable side effects. TFs play a pivotal role in the different processes, acting as regulators of gene expression. Therefore, manipulating of these TFs may effect on critical pathway which is not clear up to now and inspire adverse effects. Consequently, there is a great demand in finding non-TF genes that can help plants to cope with both biotic and abiotic stresses.

Thaumatin like proteins (*TLP*s) are promising candidates for this purpose. There is a wide distribution of these proteins in both plants and animals (Shatters et al., 2006) (Table 1.). Rajam et al. (2007) demonstrated that leaf extract of tobacco transgenic plants with *TLP* effectively inhibited the mycelial growth of both *Pythium aphanidermatum* and *Rhizoctonia*

Table 1. Thaumatin-like protein sequences (total number in brackets) and their GenBank accession numbers or gene locus in representative species belonging to the plant, animal, and fungal kingdoms

Plant
Arabidopsis thaliana (28)
NP_173261, NP_173365, NP_173432, NP_177182, NP_177503, NP_177640, NP_177641, NP_177642, NP_177708, NP_177893, NP_179376, NP_180054, NP_180445, NP_192902, NP_193559, NP_194149, NP_195324, NP_195325, NP_195579, NP_195834, NP_197850, NP_198644, NP_198818, NP_568046, NP_973870, NP_001031064, NP_001031809, NP_001078513
Oryza sativa (31)
NP_001041821, NP_001044756, NP_001049473, NP_001049531, NP_001049533, NP_001050827, NP_001050832, NP_001050833, NP_001052130, NP_001054348, NP_001058421, NP_001059476, NP_001062246, NP_001063785, NP_001063786, NP_001064158, NP_001064159, NP_001064589, NP_001067069, NP_001067073, NP_001067074, NP_001067331, NP_001067332, NP_001067333, NP_001067334, NP_001067335, NP_001067336, NP_001067337, NP_001068535, NP_001068536, NP_001068554
Picea glauca (13)
DR548648, DR550004, DR551624, DR552739, DR555245, DR566085, DR573433, DR580767, DR582629, DR584727, DV996551,EX409023, EX409554
Pinus monticola (10)
GQ329659 to GQ329668
Physcomitrella patens subsp. *Patens* (6)
XP_001765997, XP_001769333, XP_001784610, AJ566726, BJ186638, FC369913
Zea mays (7)
NM_001155967, NM_001156935, NM_001153537, NM_001156271, NM_001157865, NM_001157648 NM_001155584
Triticum aestivum (5)
X58394, AF442967, AF389884, AF384146, X97687
Animal
Acyrthosiphon pisum (6)
XP_001942530, XP_001942572, XP_001942718, XP_001942779, XP_001942788, XP_001951906
Tribolium castaneum (5)
XP_968724, XP_969010, XP_975156, XP_975166, XP_975175
Locusta migratoria (3)
CO821337, CO821344, CO849988
Caenorhabditis elegans (6)
NP_500747, NP_500748, NP_502360, NP_502361, NP_502362, NP_507263

These sequences were searched out by BLAST through the available genomes at GenBank, and through the Gene Index Project data sets at Dana-Farber Cancer Institute (Liu et al. 2010)

solani in vitro. Interestingly, overexpressing of *TLP* showed enhanced resistance to late blight, sheath blight, and Fusarium head blight (FHB) in transformed plants such as potato (Jin, 2001), rice (Datta et al., 1999), and spring wheat (Pritsch et al., 2000). Xing et al., 2008 discovered that *Ta-TLP* (*Triticum aestivum TLP*) is one of the most important genes for improving the wheat powdery mildew resistance pathway (Xing et al., 2008). More recently, Wang et al. (2010) identified *TLP*s overexpression gene during resistance response to the stripe rust fungus (Wang et al., 2010). Liu et al., 2010 found that besides the previously identified role of *TLP*s in pathogen resistance, they can play a prominent role in cold tolerance, salinity and drought resistance. It is not still clear how *TLP*s confer resistance against abiotic stress (Liu et al., 2010). However, it seems that *TLP*s can be proposed as the new reliable non-TF genes for inducing resistance against the broad range of biotic and abiotic stresses.

Up to now, *TLP*s have been classified as a member of pathogenesis related proteins (PRs) (Xing et al., 2008). According to amino acid sequence similarities, serological relationships, and biochemical properties, PR proteins are characterized into 17 groups (Van Loon and Van Strien, 1999). PR-5 proteins family members are referred to thaumatin-like proteins (*TLP*s) (Futamura et al., 2006). *TLP*s are plant-defense proteins which microbial attack, chemical elicitors such as salicylic acid (SA), methyl jasmonate (MJ) and ethylene can induce their expression (Kitajima and Sato, 1999).

Although recent studies have unraveled some of the key role of *TLP*s, many questions related to mode of regulation and signaling networks are still remained unanswered.

Large scale genome sequencing projects, by producing thousands of megabases each year, provide new opportunities for studying molecular function of unknown genes or discovering the novel genes which possibly interact with a certain gene through bioinformatics tools such as EST analysis, promoter analysis and *in silico* chromosome walking. For instance, EST analysis unraveled cytosolic isoforms of glutathione reductase as the main isofor responding to stress condition. *MtATP6* promoter analysis suggested abscisic acid-mediated signaling, energy metabolism, and stress response for this gene based on cis acting elements such as ABRE, MYC, MYB and W-boxes identified on its promoter (Abolimoghadam et al., 2012). In our previous study, we employed promoter analysis to reveal some TLPs and OLPs novel isoforms involving in response to both biotic and abiotic stresses (Deihimi et al., 2012). In this study we employed *in silico* chromosome walking as computational analysis.

In plant genomes, data bases like Plant Genome Duplication Database (Tang et al., 2008) and Phytozome (http://www.phytozome.net/) have provided the visualized user-friendly environment for finding the genes around a specific locus and comparative study of this gene block between various genomes. This method is based on the fact that regarding multigenic nature of a lot of biological events,

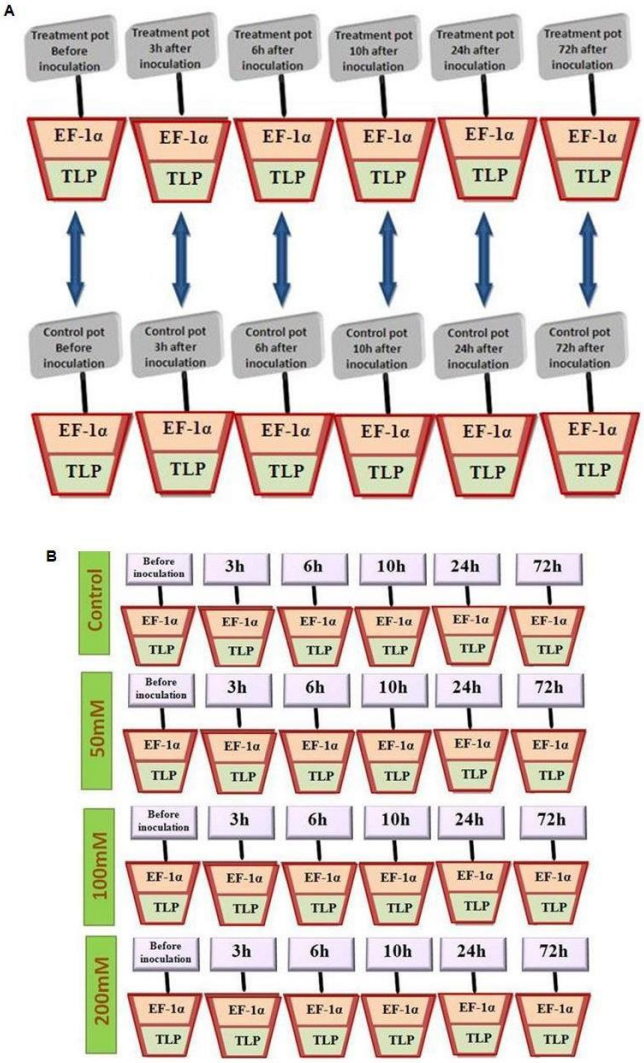

Fig 1. Schematic diagram of biotic and abiotic experiments. A. Schematic diagram of fungi stress experiment. *EF-1α* is the reference gene used in PCR reactions and *TLP* is the thaumatin like protein gene which is the gene of interest. The experiment has been conducted at 0h (control), 3h, 6h, 24h and 72h after inoculation. B. Schematic diagram of salt stress. *EF-1α* is the reference gene used in PCR reactions and *TLP* is the thaumatin like protein gene which is the gene of interest. The experiment has been conducted at 0h (control), 3h, 6h, 24h and 72h after inoculation. salt stress was conducted at 3 level of 50mM, 100mM and 200mM NaCl.

the involved genes in a same function are locating near each other during the evolution and this structure is kept between different genomes with some alteration (Hurst et al., 2004). This architecture allows the cell to spend less energy for switching on/off of the genes with easy access. Even, when the genes on a region of a chromosome are poorly identified, we can still find the possible genes around the gene of interest by downloading the raw sequence of that region and searching for open reading frames. Real interaction between the surrounding genes with the gene of interest can be further confirmed by Real-time PCR analysis.

In short, *TLP* gene expression and biological function are still poorly understood (O'Leary et al., 2007). In this study, *TLP* expression against *Septoria tritici* infection and salt stress was analyzed by Real Time PCR techniques in fungal resistant, susceptible and also salt resistant wheat genotypes to clarify the expression pattern of *TLP* in compatible and incompatible interactions. In addition, introducing the novel *TLP* isoform, that can play significant role in both fungal and salt stresses, for the first time, make this study typical. This approach can be a new venture in genetic engineering and gene transformation. Hence, we can confer resistance to both biotic and abiotic stresses by just one transformation. Applying new bioinformatics techniques including study the *TLP* adjacent genes on the chromosome, and *in silico* chromosome walking, are another innovation of this study, which were carried out to illustrate molecular function and regulatory mechanisms of *TLP*.

Results

Expression profile of wheat TLP gene in response to Septoria tritici

110bp cDNA fragment was amplified using RT-PCR. The sequence was obtained by VectorNTI software. BLASTX analysis of 110bp sequence showed high similarity (100%) to *TaTLP* (GenBank accession no. X58394). The accumulation of *TLP* transcripts in wheat leaves infected with *Septoria tritici*, was validated using Real Time RT-PCR assay in different time courses (3h, 6h, 10h, 24h and 72h after inoculation). Our results showed the significant difference in relative expression of *TLP* gene between the *S.tritici*-susceptible and resistant cultivar of wheat (Figure 2). In sampling time points, the maximum level of mRNA, accumulated at 6h after inoculation in both resistant and susceptible cultivars, while the expression of transcript in resistant genotype was almost 1.5 fold higher compared with susceptible genotype. In the incompatible interaction, *TLP* mRNA accumulation decreased from 6 to 24h after inoculation, but still higher than transcript expression in the control tissue except at 24h after inoculation. At 72h after inoculation, slight increase occurred in *TLP* transcript expression in compared with 24h after inoculation time point. From 6 to 72h after inoculation, in the compatible interaction, *TLP* expression decreased even lower than the control tissue. Beginning of *TLP* induction in resistant cultivar occurred at 3h after inoculation. Quite opposite to resistant genotype, *TLP* over-expression in susceptible cultivar started 6h after inoculation (Figure 2). However the levels of mRNA accumulation in compatible interaction were much lower than in incompatible reaction.

Expression profile of wheat TLP gene in response to abiotic stress

In this experiment, qRT-PCR applied to test whether the expression of *TLP* was upregulated by different levels of NaCl in different time courses. Leaves of Mahuti (salt resistant cultivar of wheat) treated by different levels of NaCl (50mM, 100mM and 200mM). The results suggested that gene expression of *TLP* (AC: X58394), increased dramatically at 3h after salt stress with 50mM NaCl. However transcript accumulation of *TLP* decreased sharply at 6h in 50mM NaCl treatment but still, it was higher than control tissue. *TLP*'s mRNA in 100mM NaCl, accumulated gradually from 3 and peaked at 72h. In 200mM NaCl, *TLP* expression peak was at 10h after salt stress, but the *TLP* gene expression was lower than transcript expression in the control tissue at all times of sampling (Figure 3).

Table 2. Comparison between TLP expression in susceptible and resistant cultivar (p=0.05).

Wheat cultivars	TLP expression
T.aestivum cv. Falat (susceptible cultivar)	1.18 [b]
T.aestivum cv. Marvdasht (resistant cultivar)	3.06 [a]

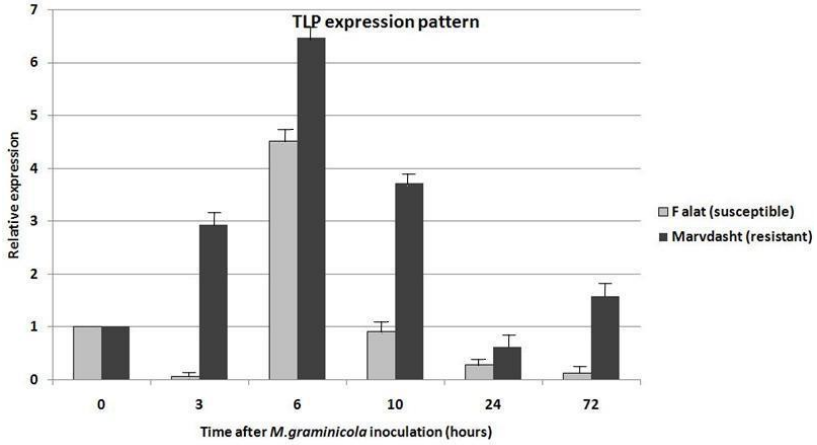

Fig 2. Quantitative RT-PCR analysis of the expression profile of *TLP* in wheat leaves after inoculation with *Septoria tritici* (infected) and before inoculation (control) from the resistant and susceptible cultivars. Wheat cultivars used are Marvdasht (*S.tritici*-resistant wheat genotype) and Falat (*S.tritici*-susceptible wheat genotype). Leaf tissue for the 0 h time point was collected just prior to inoculation. Data are normalized to wheat Elongation factor 1α (*EF-1α*) gene expression Level (to the calibrator, Control 0h, taken as 1.00).

Statisical analysis of *TLP* expression data reached by Real Time PCR

T-Test analysis of Real-Time PCR data comparison showed that there is significant difference between time points after inoculation (P=0.05) in both susceptible (Falat) and resistance (Marvdasht) cultivar (Table 2). Time points mean of *TLP* gene expression based on Real time PCR analysis for resistant cultivar is 3.06 whereas for susceptible cultivar is 1.180 and T-Test confirmed this difference at P=0.05 (Table 2).

In silico chromosome walking of TLPs homologues in Arabidopsis thaliana

In order to find co-expressive proteins near each isoform the *in silico* chromosome walking carried out for 5 loci of *TLP*s in *Arabidopsis thaliana* (Figure 4). Among all proteins identified near these 5 loci (AT5G24620, AT1G75030, AT1G18250, AT1G73620, AT5G02140), the role of 3 of them in response to salt stress has been proved (Table 3). Since the genes which are near each other can act together to control an event, finding the salt-responsive genes around can lead us to discover the genes with dual role in salt and fungal stresses. As presented in Table 4 and Figure 4, both *in silico* chromosome walking and our previous results by promoter analysis (Deihimi et al., 2012) revealed 3 loci of *TLP*s including AT1G75030, AT1G18250, and AT1G73620 that can be suggested as candidate homologs for responding to both fungal and salt stresses.

Discussion

In 1970, tobacco infected with tobacco mosaic virus (TMV) expressed PR proteins. After that, numbers of PR proteins had been discovering in a wide variety of plant species such as PR1 (unknown), PR2 (b-1,3-glucanase), PR3 (chitinase type I, II, IV, V, VI, VII), PR5(osmotins and thaumatin like proteins), PR8 (chitinase type III)and PR10 and so on (Zhang et al., 2012). B-1, 3-glucanases involve in pathogen defence are classified as PR2 (Saikia et al., 2005). PR3 is named as chitinase which is regulated by wounding, methyl jasmonate, Ethylene and Gibberellin (Wu and Bradford, 2003). Thaumatin-like Proteins belong to PR5 proteins. Over the past decade, *TLP*s have been discovered in a wide range of organisms (Shatters et al., 2006). Van Loon et al. 2006 identified that plants are induced by pathogen invasion, drought, salt, wounding and cold hardiness (Van Loon et al., 2006). There is limited information about *TLP*s mechanism in response to both biotic and abiotic stresses. In this study we took advantage of real time PCR technique and bioinformatics tools in order to reveal *TLP*s pathway clearly. Real time PCR technique is a powerful tool in order to reveal the gene expression pattern. In our previous study we suggested the presence of salt induced *Ta*SOS1 and HKT 1;5 on genome A and D of wheat ancestors by this powerful technique (Ramezani et al., 2012; Zamani et al., 2012).

In our assay of *TLP* gene expression in leaves of wheat plants, and their possible role in response to wide range of stresses, 110bp cDNA was isolated. *S.tritici* and different levels of salinity (50mM, 100mM, 200mM NaCl) applied as biotic and abiotic stresses to confirm the idea.

Here we found that in response to *S.tritici*, *TLP* transcript level begin accumulating so early at 3h after inoculation and reach the maximum amount at 6h after fungal inoculation in both resistant and susceptible cultivar of wheat. This rapid induction is rare in response to other fungi. For instance, in *Fusarium graminearum* infected wheat spike tissue, some PR genes reach the maximum level of induction at 48h after inoculation (Pritsch et al., 2000). Wang et al. 2010 revealed that the expression of *Ta*PR5 was induced at 48 hour post inoculation (Wang et al., 2010). But Ray et al. 2003

Table 3. Proteins near *TLP*s that are responsible for salt stress tolerance.

protein	function
calcium-binding protein	Response to Salt stress
heat shock protein-binding	Response to Salt stress
brassino steroid insensitive	Response to Salt stress

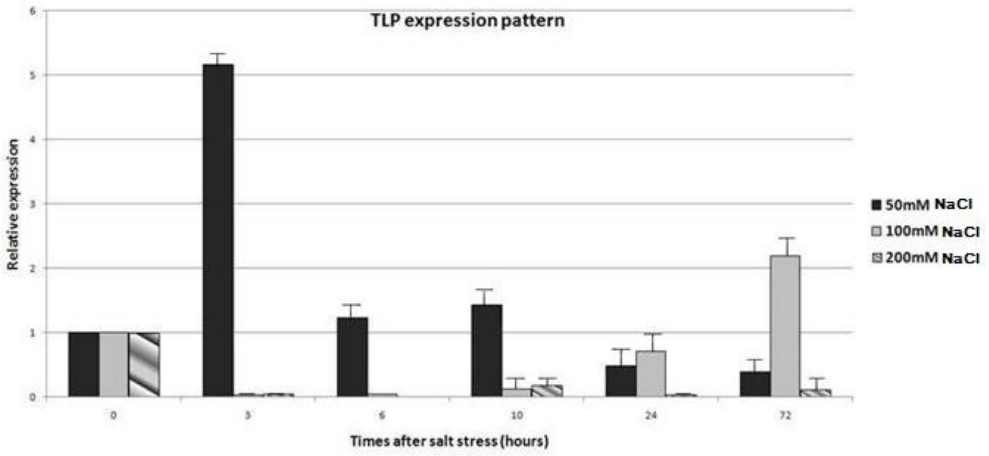

Fig 3. Quantitative RT-PCR analysis of the expression profile of *TLP* in wheat leaves during salt stress. Three levels (50mM, 100mM, 200 mM) of salt stress applied in wheat leaf tissue of the resistant cultivar of wheat "Mahuti". Leaf tissue for the 0 h time point was collected just prior to salt application. Data are normalized to wheat Elongation factor 1α (*EF-1α*) gene expression level (to the calibrator, Control 0 h, taken as 1.00). The mean expression value was calculated with three independent replicates.

confirmed our results and found rapid accumulation of *TLP* as early as 3h after inoculation of wheat by *S.tritici* (Ray et al., 2003). It can be realized that, defense responses may act through different pathway against diverse pathogen and plants. Salicylic acid (SA), methyl jasmonate(MJ), ethylene(ET), and abscisic acid (ABA) are the pathway by which thaumatin like protein genes are regulated in tobacco (Stintz et al., 1991). In *Arabidopsis thaliana* the expression of *TLP* was controlled by SA and 2, 6-dichloroisonicotinic acid (INA). The *TLP* expression in *Malus hupehensis* in contrast with tobacco, arabidopsis and rice is in response to SA, MeJA and 1-aminocyclopropane-1-carboxylic acid (ACC) (Zhang et al., 2010). Hence, differential expression of *TLP* in distinct resistant cultivars of wheat may indicate the diverse pathway by which *TLP*s are regulated.

Previous studies have showed that in wheat, *S.tritici* conidia commence germinating on leaf surface about 2 h after inoculation (Cohen and Eyal, 1993). Consequently, materials from non-germinated spores and spore germlings of *S.tritici* in an extracellular matrix (ECM) may induce the rapid responses of host (Duncan and Howard, 2000). Cohen and Eyal (1993) suggested that growth of sporogenous hyphal biomass might be slow down by rapid induction of defense mechanism. In the resistance interaction, hyper sensitive response (HR) caused cell death as early as 24h after inoculation (Wang et al., 2010; Kang et al., 2002). Hence, the pathogen induced accumulation of this *TLP* is prerequisite to HR.

Differential transcript levels of *TLP* genes between the compatible and incompatible interactions in this study indicated that their constitutive expression contributes to the resistant phenotype. Pritsch et al. (2000) reported that *TLP* genes are induced to higher levels in incompatible than compatible interactions, while in this study we found that level of *TLP* expression in incompatible interaction is constantly higher than compatible interaction. Discovering differential expressed genes involve in responding to the pathogen may help recognition of cellular processes activated or repressed during the early phase of host-pathogen interactions (Ray et al., 2003). It should be noticed that PR gene families play key roles in the resistance process and their expression in transgenic plants imparts significant disease resistance (Datta et al., 1999).

The most outstanding point in this research is the notification of all analysis to the bifunctional role of *TLP*s in response to both biotic and abiotic stresses. In this study we report the isoform of *TLP* which may play significant role in both salt and fungi stresses. This isoform of *TLP* (AC: X58394) can be introduced as a novel isoform which has bifunctional role in both biotic and abiotic stresses. Exposure of wheat leaves to 200mM NaCl, resulted in no induction of *TLP*, but in 100mM stress of salt which is still high salinity stress, the induction occurred at 72h after stress. Despite those 2 levels of stress, at 50mM of NaCl rapid induction of *TLP* happened at 3h after stress. Differential induction observed in response to different amount of salt.

Based on upregulation of *TLP* (AC: X58394) observed in response to salt stress, it may be the isoform which can be a precious choice for a single transformation in order to induce resistant to both biotic and abiotic stresses. However it needs more study to confirm this result.

Efficiency of in silico analysis

In this study, bioinformatics tools for the first time explained the bifunctional role of *TLP* more obviously. Using bioinformatics tools to reveal *TLP*s mechanism more evidently, is the strong point of this research. In previous assay coexpressed gene analysis and promoter analysis confirmed our *in silico* chromosome walking results and

Table 4. Proposed *TLP* isoforms that can be activated by both biotic and abiotic stresses based on 2 bioinformatics tools.

Organism	Accession number	Method
Arabidopsis thaliana	AT5G24620	*In silico* chromosome walking
Arabidopsis thaliana	AT1G75030	*In silico* chromosome walking/*In silico* promoter analysis
Arabidopsis thaliana	AT1G18250	*In silico* chromosome walking/*In silico* promoter analysis
Arabidopsis thaliana	AT1G73620	*In silico* chromosome walking/*In silico* promoter analysis
Arabidopsis thaliana	AT1G77700	*In silico* promoter analysis
Arabidopsis thaliana	AT4G36010	*In silico* promoter analysis
Arabidopsis thaliana	AT4G38660	*In silico* promoter analysis
Arabidopsis thaliana	AT5G02140	*In silico* promoter analysis
Arabidopsis thaliana	AT5G40020	*In silico* promoter analysis
Oryza sativa	OS04G0689900	*In silico* promoter analysis
Oryza sativa	OS10G0412700	*In silico* promoter analysis

identified some novel isoforms of *TLP* could response to both biotic and abiotic stresses (Deihimi et al., 2012).
Our approach (responding to different sorts of stresses) opens a new opportunity in genetic engineering.
Previous researches show that position of genes in genomes does not occur randomly (Hurst et al., 2004; Smon and Duret, 2006; Li et al., 2006). For instance, in human genome housekeeping genes and genes that contribute in the same pathway tend to be positioned adjacent to each other in the genome (Hurst et al., 2004). Studies indicate that in *Drosophila*, nematode and yeast changing in organization of adjacent genes could disturb the co regulated transcription over these genes. Hence, the co expression of adjacent genes is crusial (Kruglyak and Tang, 2000; Spellman and Rubin, 2002; Trinklein et al., 2004). Tsai et al. (2007) found that yeasts respond to environment changing efficiently, by modulating expression of adjacent genes simultaneously (Tsai et al., 2007).Therefore, our study on *in silico* chromosome walking of *TLP*s and analysis of adjacent proteins provided a new method for identifying outstanding homologes which can play active role in response to both biotic and abiotic stresses. In addition, gene networks between *TLP*s and nearby gene exposed that *TLP*s can have both biotic and abiotic stress response role. By *in silico* chromosome walking results we could suggest some *TLP* isoforms that may be active in both biotic and abiotic stresses. Table 4 shows the proposed isoforms of *TLP*s that have bifunctional activity against both biotic and abiotic stresses and also the method we used to extract these results. The most significant point which confirms our results is the same isoforme which has been suggested by both *in silico* chromosome walking and *in silico* promoter analysis (Deihimi et al., 2012). For further analysis Real Time PCR of adjacent genes, is suggested to prove our results.

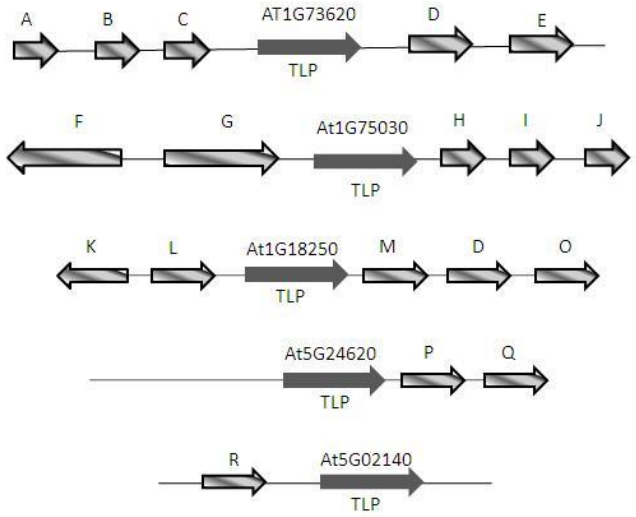

Fig 4. Gene arrangement (*in silico* chromosome walking) of different *TLP*s isoforms in *Arabidopsis thaliana*.
(A) transmembrane protein, (B) GDSL motif lipase, hydrolase family, (C) low molecular weight cyctein rich 65. (D) calcium binding protein, (E) oxidoreductase acting on the CH-CH protein. (F) acyltransferase, (G) accumulation and replication of chloroplast, (H) DNA binding transcription regulator transcription repressor, (I) methyl adenin glycosylase family protein, (J) heat shock protein binding, (K) ketose bisphosphate aldollase classII family, (L) suppressor of lin-12-like protein-related, (M) pyridoxamine 5'-phosphate oxidase, (O) purin transmembrain transporter, (P) brassino steroid insensitive, (Q) mitochhondrial import inner membrain translocase subunite Tim17/Tim 22/Tim23 family protein. (R) amino acid transporter family protein.

Materials & Methods

Plant material

Three cultivars of wheat (*Triticum aestivum* L.) were used for Real Time PCR experiments: Falat (*Septoria tritici* susceptible cultivar) Marvdasht (*Septoria tritici* resistant cultivar (Haghdel and Banihashemi, 2003) from Zarghan Agriculture Research Center (Fars, Iran) and Mahuti (one of the most salt resistant Iranian wheat cultivar) (Ghavami et al., 2004). Seeds of each cultivar were planted in pots and were germinated in a greenhouse on 18h light (27ºC) and 6h dark (18ºC). Mahuti seeds were grown in half-strength modified Hoagland nutrient solution. The nutrition solution was composed of the following: H_3BO_3, 11.5mM; $CuSO_4 \cdot 5H_2O$, 0.08mM; KNO_3, 5mM; KH_2PO_4, 1mM; $Na_2MO_4 \cdot 2H_2O$, 0.12mM; $Ca(NO_3)_2 \cdot 4H_2O$, 5mM; $MnCl_2 \cdot 4H_2O$, 4.6mM; $ZnSO_4 \cdot 7H_2O$, 0.2mM; NaFe-EDTA, 0.1mM; $MgSO_4 \cdot 7H_2O$, 2mM (Kerepesi and Gabila, 2000).

Preparation of fungal inoculum

The pathogen used for inoculation was *S. tritici*. The field treated leaves of wheat has been collected as inoculum resource. Leaves were scratched in sterile water and shaked. The solution has been spread on sterile water agar plates, and kept them in 37ºC for 24h. Germinated pycnidiospores were transformed onto fresh PDA (extraction of 360gr potato, 20gr dexterose, 16gr agar, distilled water to 1lit) for 3-4 days until

fungi colony were visible. Inoculum was standardized to 4×10^6 conidia/ml with a hemacytometer. Ten drops of Tween 20 were added as a surfactant to each liter of inoculum. Conidia were applied to the plants at the 5–6 leaf stage with hand-held sprayers. Water used for control sample.

Salt stress

Salt stress imposed 2 weeks after sowing in 3 levels of NaCl (50mM NaCl (58.45gr/lit NaCl & 9.09 gr/lit CaCl2), 100mM NaCl (116.9 gr/lit NaCl & 18.8 gr/lit CaCl2), and 200mM NaCl (233.8 gr/lit NaCl & 36.369 gr/lit CaCl2).

Experimental design for Real Time PCR experiments

Real Time PCR experiments were carried out in two tests: fungi and salt experiment. Fungi experiment performed with four treatments: non-inoculated susceptible wheat cultivar (Falat)(Control), *S.tritici*-inoculated susceptible wheat cultivar (Falat)(Infected), non-inoculated resistant wheat cultivar (Marvdasht)(Control), and *S.tritici*-inoculated resistant wheat cultivar (Marvdasht)(Infected). Salt experiment performed with 4 treatments too: non-inoculated resistant cultivar (Mahuti)(Control), 50mM NaCl stressed resistant cultivar (Mahuti)(Infected), 100mM NaCl stressed resistant cultivar (Mahuti)(Infected) and 200mM NaCl stressed resistant cultivar (Mahuti)(Infected). Leaf tissue was collected from each treatment (both salt and fungi experiment) at 0, 3, 6, 10, 24, and 72h after inoculation. Leaf tissue was frozen in liquid nitrogen and stored at −80◦ C. The inoculated seedlings were allowed to grow for assessment of disease symptoms at about 28 days. Three replications were analyzed for each treatment. In this study we inoculated and tested three plants.

Total RNA extraction and cDNA synthesis

Total RNA was extracted by RNx-Plus kit (Cinagene, Shiraz, Iran) according to the manufacturer's instructions from wheat leaves. The RNA was quantified on a spectrophotometer and visualized on a 1% agarose. Based on spectrophotometer readings, equal quantities of total RNA were pooled. First-strand cDNA for both salt and fungi experiments was synthesized from the isolated total RNA of leaf tissues harvested at different sampling times (0, 3, 6, 10, 24, and 72h after inoculation) from the resistant and susceptible cultivars with Mulv kit (Fermentas, Germany), in both salt and fungi experiment.

Real-time quantitative PCR

Primers for Thaumatin like protein (*TLP*) gene were designed based on its full cDNA sequence of Gen Bank accession number: X58394. The forward and reverse primer sequences for *TLP* Real-time PCR for both salt and fungi stresses were: 5'_GCCGCAAGCCTACCAACA-3' for forward and 5'_CGCGGTGCGACGTATAGAG- 3'_ for reverse *TLP*. In addition, a set of primers were also designed for wheat Elongation factor 1-α (*EF-1α*) (Long et al. 2010), as the normalizer of the amount of target gene, based on Gen Bank accession number: M90077.1. *EF-1α* sequences of primers were: 5'-TTTCACTCTTGGAGTGAAGCAGAT-3' for forward and 5'-GACCTCCTTGACAATTTCTTCATAA-3' for reverse. The predicted amplicon size was 110bp for *TLP* (as there were no report of X58394 expression in salt stress there was no knowledge about the amplicon size of this *TLP* during salt experiment) and 107bp for *EF-1α*. Real Time PCR reaction was performed with the PCR/RT-PCR kit Biocasy SYBR Green 1. (Cat No: bsb03l1) (Bioer, China) according to the manufacturer's instructions. Diluted cDNA samples (1:10, 1.5µl) of all treatments were used as templates in PCR reactions containing 10µl of SYBR Green PCR Master Mix and 300 nM of each primer. All reactions were set up in triplicate and subjected to real-time PCR analysis with the LineGene K FQD-48A and the SYBR Green I dye. The following cycling steps were used: initial denaturation for 2 min at 94ºC, followed by 40 cycles with 10s at 94ºC, 15s at 57ºC(for both *TLP* and *EF-1α*), and 30s at 72ºC and 30min gradient from 50-95 to obtain melting curve. No-template controls were included in each PCR plate to ensure purity of reagents and minimal carryover contamination. No-reverse-transcription (no-RT) controls were included in the PCR runs to ensure negligible contamination of the total RNA samples with genomic DNA. The schematic diagram of both salt and fungi experiment is presented in Figure 1. In this experiment *EF-1α* was used as a reference gene in PCR reactions and *TLP* is the thaumatin like protein gene which is the gene of interest. Cycle threshold values (CT) generated from LineGeneK Software Tool were employed to quantify relative gene expression using comparative 2 -ΔΔCT method (Livak and Schmittgen 2001). To balance inter PCR variation, normalization of the *TLP* gene was done via *EF-1α* gene expression and to compensate the variation in physiological changes relative quantification performed toward control samples.

Statistical analysis

Statistical analysis for data of Real Time PCR method was carried out using Minitab 14 (www.minitab.com). Two-sample T-Test carried out between the time points after fungal inoculation treatment for each cultivar. After that, Paired T-Test was done between susceptible and resistant cultivars in time points after fungal (Table 2).

In silico chromosome walking

For *in silico* chromosome walking, comparative genome analysis, in order to find the nearby genes to *TLP*, carried out using Plant Genome Database (http://chibba.agtec.uga.edu/duplication/) and PHYTOZOME databank (http://www.phytozome.net) in *Oryza sativa*, and *Arabidopsis thaliana* genomes. CLCbio software was exploited to find open reading frame (ORF) of the sequence between *TLP* and adjacent upstream and downstream genes in *Oryza sativa*, and *Arabidopsis thaliana*. This may lead to discover the possible genes which have not been reported yet. BLASTX used to predict the protein of found ORFs.

Conclusion

In conclusion the resistant cultivar of wheat we tested, rapid up-regulation of *TLP* observed during *S.tritici*. In addition we discovered induction of *TLP* during abiotic (salt) stress. Bioinformatics achievement such as *in silico* chromosome walking identified genes responsible for abiotic stresses near *TLP*s on chromosome. Although further studies are needed to confirm the mechanism of *TLP* role in response to both biotic and abiotic stresses, our finding may open a new avenue.

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