Eur J Plant Pathol (2011) 131:643–651
DOI 10.1007/s10658-011-9837-8

ORIGINAL RESEARCH

TaFLRS, a novel mitogen-activated protein kinase in wheat defence responses

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Accepted: 8 July 2011 / Published online: 26 July 2011

Abstract Plants respond to biotic and abiotic stresses through the activation and coordination of various signalling pathways. The activation often requires the phosphorylation of proteins. In this study, we have identified the wheat *TaFLRS* MAP kinase (*F*usarium and *L*eaf *R*ust *S*ensitive) gene that was upregulated in a wheat EST (expressed sequence tag) array analysis following a wheat-leaf rust interactive challenge. Our results demonstrate that *TaFLRS* is transcriptionally upregulated in incompatible interactions involving wheat and leaf rust and *Fusarium graminearum*, suggesting that this MAPK maybe involved in defence responses to these wheat pathogens. RT-PCR revealed that *TaFLRS* transcript levels are not altered by salicylic acid (SA) treatment. However, immunoprecipitation and western blotting analysis show that phosphorylation of TaFLRS at the TEY motif was enhanced by SA in the Fusarium head blight (FHB) resistant cultivar Frontana following challenge with the FHB pathogen. The role of TaFLRS MAP kinase in defence responses in wheat is discussed.

Keywords MAPK · Phosphorylation · SA · Disease resistance · Wheat

Abbreviations

FHB	Fusarium head blight
HR	hypersensitive response
MAPK	mitogen-activated protein kinase
PR gene	pathogenesis-related gene
SA	salicylic acid.

Introduction

Plant defence responses depend on recognition of invading pathogens, and this recognition may occur via gene-for-gene interactions in which a plant resistance (*R*) gene product detects the action of a cognate pathogen avirulence (Avr) factor (Dangl and Jones 2001). The wheat-leaf rust interaction, which generally follows the gene-for-gene model, remains an excellent system to study fungal diseases of plants, and many of the *Lr* genes are studied for this reason (Kolmer 1996; Jordan et al. 2006). The presence of single leaf rust resistance genes in near-isogenic lines confers resistance to avirulent races resulting in a

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hypersensitive response (HR) at the site of infection with localized cell death, and significant amelioration of disease symptoms, and this plant-pathogen interaction has been long considered a model system in the analysis of wheat defence pathways (Kolmer 1996; Jordan et al. 2006). Fusarium head blight (FHB) is also a serious disease of wheat worldwide that may cause substantial yield and quality losses (Gilbert et al. 2006). However, the nature of resistance to this disease is complex and involves several types of resistance (Gilbert et al. 2006).

To survive both disease and abiotic stresses, plants have developed elaborate mechanisms to detect external signals and respond with the appropriate physiological and morphological changes. Protein phosphorylation represents one of the major mechanisms in plant responses to a large array of biotic and abiotic stresses (Fujita et al. 2006). The phosphorylation of a protein can alter its intrinsic biological activity, subcellular location and half-life time (Xing et al. 2002). The large number of the kinases and phosphatases encoded by a single plant species further reflect the complexity of phosphorylation events and homeostasis once a plant cell is challenged by a stress signal. For example, there are approximately 1050 protein kinase genes in the Arabidopsis genome, of which 23 are mitogen-activated protein kinases (MAPKs), 9 are MAPK kinases (MAPKKs), more than 25 are MAPK kinase kinases (MAPKKKs), and 29 are calcium-dependent protein kinases (CDPKs) (Cvetkovska et al. 2005).

The MAPK cascade plays an essential role in regulating plant responses to stress. The cascade consists of MAPKs, MAPKKs and MAPKKKs. The MAPKKK activation is mediated by receptor through physical interaction and/or phosphorylation by the receptor itself, intermediate bridging factors, or interlinking MAPKKKs (Nakagami et al. 2005). The MAPKKs are activated by the MAPKKKs through phosphorylation on serine and threonine residues in a conserved S/T-X_{3-5}-S/T motif (where S represents serine, T threonine, X an amino acid). The MAPKKs are dual-specificity kinases that phosphorylate the MAPKs on threonine and tyrosine residues in the TXY motif (where T represents threonine, Y tyrosine, X an amino acid). The MAPKs phosphorylate a variety of substrates including transcription factors, protein kinases and cytoskeleton-associated proteins (Nakagami et al. 2005). On the basis of their signature activation sequences, MAPKs can be categorized into three broad subfamilies: c-Jun NH2-terminal kinases (JNKs), p38 MAPKs, and ERKs (Schramek 2002). To achieve activation, JNKs undergo dual phosphorylation at their TPY motif (where P represents proline). Members of the p38 MAPK family are primarily activated at dual phosphorylation sites in their TGY motif (where G represents glycine). ERKs, on the other hand, are activated by dual phosphorylation of a TEY motif (where E represents glutamic acid) (Schramek 2002). Activated MAPKs further regulate the expression of pathogenesis-related (*PR*) genes, HR-like cell death, systemic acquired resistance (SAR) and the expression of protective genes (Ligterink et al. 1997; Zhang and Klessig 1997; Rudd et al. 2008; Stulemeijer et al. 2007; Xing et al. 2002, 2008).

In our previous work, $tMEK2^{MUT}$, a constitutively expressed active mutant of the tomato MAPKK *tMEK2* (also named *LeMKK2*), was created by replacing amino acid S-221 and T-226 between subdomains VII and VIII with D (glutamic acid) (Xing et al. 2001) through PCR-directed mutagenesis. The negative charge of the amino acid D mimicks phosphorylation of the residue. Overexpression of $tMEK2^{MUT}$ in tomato enhanced resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* and activated different pathways in biotic stress responses (Xing et al. 2001, 2003). Transgenic wheat overexpressing tomato $tMEK2^{MUT}$ also exhibited enhanced resistance to leaf rust (*Puccinia triticina*) (Jordan et al. 2006; Fan et al. 2009). In the present study, we employ gene expression and protein phosphorylation analyses to demonstrate that wheat *TaFLRS* (*F*usarium and *L*eaf *R*ust *S*ensitive), an ERK-type MAPK, is associated with defence responses in incompatible interactions involving wheat and the pathogens *Puccinia triticina* (causal agent of leaf rust) and *Fusarium graminearum* (causal agent of Fusarium head blight). The significance of TaFLRS protein changes during the responses to pathogen attack is discussed.

Materials and methods

Bioinformatics analysis

TaFLRS gene was first identified in bread wheat *Triticum aestivum* Thatcher Lr1 in a preliminary

wheat EST (expressed sequence tag) array analysis (He and Xing. unpublished). The sequence was deposited to GenBank (GenBank accession number AY173962). The catalytic domains of TaFLRS protein were identified using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan). For sequence homology analysis, the amino acid sequence of TaFLRS was searched against the non-redundant protein database (National Center for Biotechnology Information, NIH, Bethesda, USA) using BLASTP (Altschu et al. 1997). Alignment of the selected sequences was done using ExPASy SIM-Alignment (Swiss Institute of Bioinformatics) (http://ca.expasy.org/tools/sim-prot.html).

In vitro phosphorylation assay

GST-TaFLRS protein was expressed in *Escherichia coli* according to Xing et al. (2001). Substrate phosphorylation assays contained 100 ng of TaFLRS, 1 μg of myelin basic protein (MBP), 40 mM Hepes pH 7.5, 0.5 mM dithiothreitol (DTT), 20 mM $MgSO_4$, 10 mM $MnCl_2$, 1 mM $CaCl_2$ and 1 μCi γ -^{32}P-ATP. Reactions were carried out at 30°C for 30 min. Phosphorylated products were separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories Inc, Mississauga, ON, Canada) and subject to autoradiography.

Leaf rust inoculation

Seeds of wheat cultivars Thatcher and Thatcher/Lr16 that is isogenic for the leaf rust gene *Lr16* were grown in a controlled growth chamber (ENCONAIR Technologies Inc, Winnipeg, MB, Canada) under a16 h light and 8 h darkness cycle at 22°C. Nine-day old plants were inoculated with wheat leaf rust *Puccinia recondita* race BBB (avirulent on Thatcher/Lr16) or race TJB (virulent on Thatcher/Lr16) (Kolmer1996) suspended in a light mineral oil, Bayol (Esso, Oak Bluff, MB, Canada). Twenty-five μl of a 16.6 mg uredinispores/ml of mineral oil inoculum were applied to each plant. The leaf rust culture was originally derived from a single spore isolate, which was increased, vacuum dried and stored at 4°C. Collected tissues were stored at −80°C and used for RNA extraction within 3 months.

FHB inoculation

Wheat seeds of the wheat cultivars Roblin and Frontana that are susceptible and resistant, respectively to *Fusarium graminearum*, the causal agent of Fusarium head blight, were planted in plastic cones with Metro Mix, one seed per cone, 54 plants per wheat line. Plants were grown in a controlled growth chambers (ENCONAIR Technologies Inc, Winnipeg, MB, Canada) with a 16 h light and 8 h darkness cycle at 15°C/13°C day/night temperature regime to promote tillering. The cones were placed in trays of water then transferred to Nutri-Bloom hydroponic solution after seedling emergence. The wheat spikes were inoculated at anthesis (7–8 weeks after germination). Isolate EEI 20/6 of *Fusarium graminearum* (Cuthbert et al. 2006) was used as the inoculum source. A standard conidial spore suspension was prepared in a carboxymethyl cellulose media. Spore concentration was adjusted to 50,000 conidia/μl. A spray bottle was used to apply 3–5 ml of spore suspension to wheat spikes. The plants were placed in a dark humidity chamber (at 21–22°C with 100% relative humidity) equipped with a timer set to shut off after 6 h. The plants remained in the chamber for 18 h post-inoculation. Then the entire spikes were harvested, stored at −80°C, and used for RNA extraction within 3 months.

SA treatment

Three to four leaves harvested from the 3-week-old plants were cut to ~2 cm segments and incubated in a 100 μM salicylic acid (SA) (Sigma, St. Louis, MO, USA) solution under the same growth conditions after infiltration. Leaf segments were placed adaxial side upward on the filter paper in Petri dishes so that they were just covered by the SA solution. Leaf segments treated in the same way with an equal volume of distilled water served as controls. After 24 h, 48 h and 72 h incubation in the solution at room temperature, leaf samples were placed in Falcon tubes, snap-frozen in liquid nitrogen, and stored at −80°C. RNA extraction occurred within 3 months of sampling.

RT-PCR

Total RNA was extracted from wheat leaf or spike tissues (100 mg) using TRIzol Reagent kit (Invitro-

gen, Carlsbad, CA, USA) according to manufacturer's protocol. After TRIzol extraction, deoxyribonuclease I kit (amplification grade, Invitrogen, Carlsbad, CA, USA) was used to eliminate genomic DNA contamination in the sample, and the cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) was used for cDNA synthesis according to the manufacturer's protocol. The primers used for RT-PCR were 5′-CAGGTGGCCATCAAGAAGAT-3′ and 5′-GCAGTGCTCCTCCGATAAAG-3′ for TaFLRS. A wheat actin gene (GenBank accession number AB181991) with the primer sequences 5′-GCCA CACTGTTCCAATCTATGA-3′ and 5′-TGATG GAATTGTATGTCGCTTC-3′ was used as an internal control. RT-PCR was carried out under the following conditions: 94°C for 1 min; 1 min at 94°C, 1 min at 61°C, and 1 min at 72°C for 25 cycles; and then 10 min at 72°C. The sizes of amplified PCR products for *TaFLRS* and the wheat actin gene are 237 bp and 369 bp, respectively.

Protein extraction and immunoprecipitation

Wheat protein was extracted from leaves (100 mg) either in extraction buffer (20 mM Tris–HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM $NaVO_3$, 10 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5% Nonidet P-40, and 1% Triton X-100), or using TRIzol Reagent kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Protein concentration in tissue extracts were determined using Coomassie blue dye binding method with the Bradford reagent and bovine serum albumin (BSA) as the standard.

A GST-TaFLRS polyclonal antibody used for immunoprecipitation was raised in rabbit. GST-TaFLRS was injected into rabbit, which was followed by a second injection four weeks after the first injection. Blood is collected from the rabbits two weeks later and the antiserum was isolated by centrifugation, followed by antibody purification by IgG affinity chromatography (Sigma, St. Louis, MO, USA). For immunoprecipitation, 15–30 μg of isolated proteins was boiled for 5 min in 2 x immunoprecipitation buffer (20 mM sodium phosphate, pH 7.2, 300 mM NaCl, 2 mM EDTA, and 3% Triton X-100), followed by centrifugation for 10 min at 15,000*g*. An equal volume of 2 x immunoprecipitation buffer was added to the supernatant together with 5 μl of antibodies. After 10 min of gentle mixing, the mixture was incubated overnight at 4°C. An equal volume of 10% pre-washed protein A-agarose (Sigma, St. Louis, MO, USA) was added. The samples were incubated at room temperature for 2 h before centrifugation for 4 min at 15,000*g*. The supernatant was discarded and the pellet was washed three times with 1 ml of immunoprecipitation buffer by mixing and centrifugation at 15,000*g*. The pellet was finally resuspended in SDS-PAGE sample buffer.

SDS-PAGE and immunoblotting with anti-phospho-p44/42 MAP kinase antibody

After centrifugation, protein samples were used for electrophoretic separation (Mini-PROTEAN 3 System, Bio-Rad Laboratories Inc, Mississauga, ON, Canada). After electrophoresis, proteins were transferred onto PVDF membranes by wet transfer with pre-chilled transfer buffer containing 25 mM Tris (pH 8.5), 192 mM glycine, and 20% v/v methanol at 4°C for 120 min at 70 V. For antibody detection, the primary antibody used was phospho-p44/42 MAP kinase (Erk 1/2) antibody at 1:1000 v:v (Cell Signaling Technology, Danvers, MA, USA). After overnight incubation with the primary antibody, blots were washed with TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) (5 min × 3) and then incubated at room temperature for 1 h with 1:2000 v:v dilution of the secondary antibody (anti-rabbit IgG, horse radish peroxidase-linked) (Cell Signaling Technology, Danvers, MA, USA). The target protein on the PVDF membrane was detected using an enhanced chemiluminescence (ECL) system containing 1x LumiGLO reagent and 1x peroxide (Cell Signaling Technology, Danvers, MA, USA). The membrane was scanned using FluorChem Q imaging system (Alpha Innotech Cooperation, Santa Clara, CA, USA). To confirm equal loading in each lane of the blot, immunoprecipitated proteins were run on SDS-PAGE and proteins were visualized by staining the gel for 10 min with Coomassie blue solution (0.25% Coomassie Brilliant Blue R, 50% methanol and 7.5% acetic acid) followed by destaining for 30 min with destain solution (20% distilled water, 20% acetic acid, 60% methanol).

Results

Characterization of TaFLRS

A full length cDNA of 1212 nucleotides and the deduced amino acid sequence of 403 amino acids encoding TaFLRS show that TaFLRS has an ERK MAPK signature motif TEY at amino acids 220–222 (Fig. 1a). Using InterProScan Sequence Search tool, amino acids 62–357 correspond to a protein kinase domain. Amino acids sequence alignment of TaFLRS and MAP kinase 6 from *Oryza sativa* (japonica cultivar) (NM_001063381) indicates a 91.4% identity in a 399 residue-overlap (Fig. 1b). TaFLRS phosphorylated myelin basic protein (MBP) in the in vitro assay, demonstrating kinase activity of the TaFLRS protein (Fig. 1c). These results indicate that TaFLRS is a MAPK and is able to phosphorylate a substrate protein.

Upregulation of TaFLRS transcription by leaf rust challenge

When inoculated with race BBB of *P. triticina* which carries the *Avr* gene product recognized by *Lr16*, the near-isogenic wheat line Thatcher/*Lr16* showed no pustules 9 days following inoculation, while abundant pustules were observed when Thatcher/*Lr16* was inoculated with race TJB which lacks an *Avr* gene product recognized by *Lr16* (Fig. 2a). No symptoms were observed on mock-inoculated seedlings (Fig. 2a). *TaFLRS* was transcriptionally upregulated 1.5 h after race BBB challenge, and at 4 h the *TaFLRS* transcriptional level returned to the control level (Fig. 2b). There was no significant change upon race TJB challenge (Fig. 2b).

Upregulation of TaFLRS transcription by Fusarium head blight challenge

Wheat cultivars Frontana (FHB resistant) and Roblin (FHB susceptible) were challenged by *Fusarium graminearum* the causal agent of Fusarium head blight. The transcript level of TaFLRS gene in wheat head was undetectable in both cultivars, 0 and 6 h following FHB inoculation and the upregulation of TaFLRS was detected only in Frontana at 24 h following inoculation (Fig. 3).

Phosphorylation of TaFLRS by SA treatment

RT-PCR analysis revealed that there were no changes of *TaFLRS* transcript levels when Frontana and Roblin leaves were treated with SA (data not shown). The possibility of regulation through protein phosphorylation at TEY motif in the TaFLRS protein was studied by examining the in vivo phosphorylation pattern of TaFLRS after SA treatment. Following immunoprecipitation with TaFLRS polyclonal antibody, proteins were subjected to immunoblotting using phospho-p44/42 MAPK (Erk1/2) antibody (anti-ERK-pTEpY) that specifically detects the dual phosphorylation on T and Y residues that is an essential feature of their post-translational activation. Upon SA treatment for 48 h, the level of phosphorylation was higher for TaFLRS in the resistant cultivar Frontana compared to the susceptible cultivar Roblin where no change was observed (Fig. 4). The result may suggest that the ERK-type MAPK TaFLRS in wheat resistant cultivar could be involved in the SA signalling pathway at the post-translational level. This anti-ERK-pTEpY antibody was used previously and has detected phosphorylation changes of the wheat MAPKs (TaMPK3 and TaMPK6) during pathogen attacks in wheat (Rudd et al. 2008).

Discussion

MAPK pathways play a central role in defence response to disease and abiotic stresses. In the present study, we report the characterization of the *TaFLRS*, a MAPK in wheat identified in a wheat EST array analysis following inoculation with *P. triticina* (He and Xing, unpublished). *TaFLRS* is a TEY motif MAPK that is transcriptionally upregulated in incompatible interactions in wheat following inoculation by *P. triticina* leaf rust and *F. graminearum*. While *TaFLRS* transcript levels were not altered by the SA treatment, the phosphorylation of TaFLRS at the TEY motif was enhanced by SA in FHB resistant cultivar Frontana following challenge with the *F. graminearum*. These results suggested that TaFLRS MAPK is involved in generalized defence responses to wheat pathogens. In a recent study we reported that *tMEK2* (*LeMKK2*), a tomato MAPKK, is a key component in the defence pathways against pathogen attacks, and the wheat transformed with *tMEK2* acquired partial

Fig. 1 **a** Nucleotide sequence and the deduced amino acid sequence of *TaFLRS*. The TEY motif is shaded and the highly conserved catalytic domain of protein kinases is underlined. The stop codon is indicated with an asterisk. **b** Amino acid sequence alignment of wheat *TaFLRS* (AY173962) and MAP kinase 6 from *Oryza sativa* (japonica cultivar) (NM_001063381). There is a 91.4% identity in 399 overlapping residues. **c** In vitro TaFLRS kinase assay. Recombinant TaFLRS proteins were incubated in vitro with MBP followed by SDS-PAGE and transfer onto PVDF membranes. Reaction without the added recombinant TaFLRS proteins was used as control. Band for phosphorylated MBP was visualized by autoradiography

a

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   1 atggacgccggcggggcgcagccgccggactcggagatggcggaggccggtgcgggcgct
     M  D  A  G  G  A  Q  P  P  D  S  E  M  A  E  A  G  A  G  A   20
  61 gcggcggcggcggggacggcgccgggcggcgcgatggataacatccaggccacgctcacc
     A  A  A  A  G  T  A  P  G  G  A  M  D  N  I  Q  A  T  L  T   40
 121 cacggcgggaggttcatccagtacaacatcttcggcaacgtcttcgaggtcaccgccaag
     H  G  G  R  F  I  Q  Y  N  I  F  G  N  V  F  E  V  T  A  K   60
 181 tacaagccccccatcctccccatcggcaagggcgcctacggcatcgtctgctccgcgctc
     Y  K  P  P  I  L  P  I  G  K  G  A  Y  G  I  V  C  S  A  L   80
 241 aactccgagacgggggagcaggtggccatcaagaagatcgccaacgccttcgacaacaag
     N  S  E  T  G  E  Q  V  A  I  K  K  I  A  N  A  F  D  N  K  100
 301 atcgacgccaagcgcacgctgcgggagatcaagctgctccgccacatggaccacgagaat
     I  D  A  K  R  T  L  R  E  I  K  L  L  R  H  M  D  H  E  N  120
 361 attgttgcaataagggatattatacctcctgcacaaaggactgcattcaatgatgtctat
     I  V  A  I  R  D  I  I  P  P  A  Q  R  T  A  F  N  D  V  Y  140
 421 attgcatatgaattgatggacaccgatctgcatcaaattattcgctcaaatcaagcttta
     I  A  Y  E  L  M  D  T  D  L  H  Q  I  I  R  S  N  Q  A  L  160
 481 tcggaggagcactgccagtatttcctttatcagatccttcgtggcttgaagtacatacat
     S  E  E  H  C  Q  Y  F  L  Y  Q  I  L  R  G  L  K  Y  I  H  180
 541 tcagcaaatgttctccaccgagacttgaagcctagcaatcttcttttgaatgcaaactgt
     S  A  N  V  L  H  R  D  L  K  P  S  N  L  L  L  N  A  N  C  200
 601 gacctaaaaatttgcgattttgggcttgctcgtaccacctcagaaactgattttatgacc
     D  L  K  I  C  D  F  G  L  A  R  T  T  S  E  T  D  F  M  T  220
 661 gagtatgttgtgacaagatggtacagggcaccagagcttttgttgaactcctccgaatac
     E  Y  V  V  T  R  W  Y  R  A  P  E  L  L  L  N  S  S  E  Y  240
 721 actgcagcaattgatgtgtggtctgtgggctgtatatttatggaactgatggaccggaaa
     T  A  A  I  D  V  W  S  V  G  C  I  F  M  E  L  M  D  R  K  260
 781 ccgttgtttccaggaagagaccatgtccatcagctacgtctactaatggaggttttccct
     P  L  F  P  G  R  D  H  V  H  Q  L  R  L  L  M  E  V  F  P  280
 841 tgtggtatttcctcgttgcagctcattggaacaccaaatgaggccgatttggattttgta
     C  G  I  S  S  L  Q  L  I  G  T  P  N  E  A  D  L  D  F  V  300
 901 aatgaaaatgcaagaagatatatccgccaacttccccgtcatgcaaggcaatcattatct
     N  E  N  A  R  R  Y  I  R  Q  L  P  R  H  A  R  Q  S  L  S  320
 961 gagaagtttccacatgttcacccttcagcaattgacttggttgaaaagatgctgactttc
     E  K  F  P  H  V  H  P  S  A  I  D  L  V  E  K  M  L  T  F  340
1021 gatcctagacagagaataacagttgaaggcgcacttgcgcatccttacctggcatcgctg
     D  P  R  Q  R  I  T  V  E  G  A  L  A  H  P  Y  L  A  S  L  360
1081 catgatataagtgatgagccagtctgcacgatgcccttcagctttgacttcgagcagcat
     H  D  I  S  D  E  P  V  C  T  M  P  F  S  F  D  F  E  Q  H  380
1141 gcgttgtcggaagaacaaatgaaggatctaatccaccaggagggcatcacgttcaaccct
     A  L  S  E  E  Q  M  K  D  L  I  H  Q  E  G  I  T  F  N  P  400
1201 gattaccagtag
     D  Y  Q  *                                                  403
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b

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AY173962,      5 GAQPPDSEMAEAGAG-----AAAAAGTAPG-GAMDNIQATLTHGGRFIQYNIFGNVFEVT
NM_0010633     4 GAQPPDTEMAEAGGGQQPPAAAAAAAGAGAGAGMMENIQATLSHGGRFIQYNIFGNVFEVT
                 ****** ****** *     ******   * * * ****** ******************

AY173962,     59 AKYKPPILPIGKGAYGIVCSALNSETGEQVAIKKIANAFDNKIDAKRTLREIKLLRHMDH
NM_0010633    64 AKYKPPILPIGKGAYGIVCSALNSETGEQVAIKKIANAFDNKIDAKRTLREIKLLRHMDH
                 ************************************************************

AY173962,    119 ENIVAIRDIIPPAQRTAFNDVYIAYELMDTDLHQIIRSNQALSEEHCQYFLYQILRGLKY
NM_0010633   124 ENIVAIRDIIPPPQRNSFNDVYIAYELMDTDLHQIIRSNQALSEEHCQYFLYQILRGLKY
                 ************ **  ******************************************

AY173962,    179 IHSANVLHRDLKPSNLLLNANCDLKICDFGLARTTSETDFMTEYVVTRWYRAPELLLNSS
NM_0010633   184 IHSANVLHRDLKPSNLLLNANCDLKICDFGLARTTSETDFMTEYVVTRWYRAPELLLNSS
                 ************************************************************

AY173962,    239 EYTAAIDVWSVGCIFMELMDRKPLFPGRDHVHQLRLLMEVFPCGISSLQLIGTPNEADLD
NM_0010633   244 EYTAAIDVWSVGCIFMELMDRKPLFPGRDHVHQLRLLME----------LIGTPNEADLD
                 ****************************************          ***********

AY173962,    299 FVNENARRYIRQLPRHARQSLSEKFPHVHPSAIDLVEKMLTFDPRQRITVEGALAHPYLA
NM_0010633   294 FVNENARRYIRQLPRHARQSFPEKFPHVHPLAIDLVEKMLTFDPRQRITVEGALAHPYLA
                 ********************  ******** **************************

AY173962,    359 SLHDISDEPVCTMPFSFDFEQHALSEEQMKDLIHQEGITFNPDYQ
NM_0010633   354 SLHDISDEPVCSSPFSFDFEQHALSEEQMKDLIYQEGLAFNPDYQ
                 ************  ********************* ***  ******
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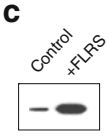
c



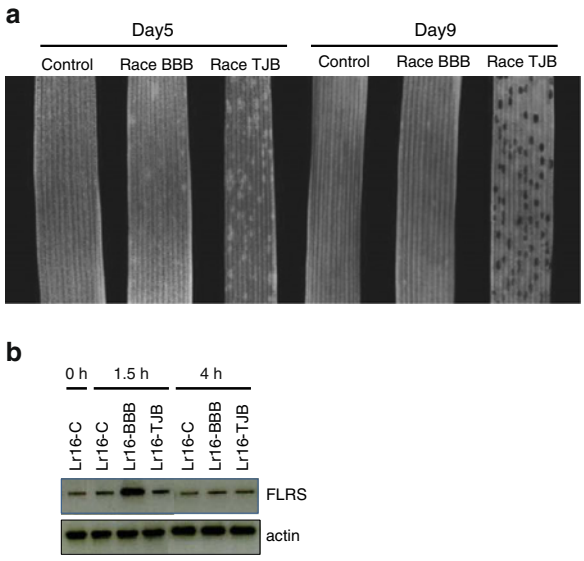


Fig. 2 **a** Phenotypic reaction of wheat near-isogenic line Thatcher Lr16 to mock-inoculation with oil (control) or inoculation with avirulent race BBB or virulent race TJB of *Pucinia triticina*. **b** Transcriptional upregulation of TaFLRS gene in wheat upon leaf rust challenge. Wheat near isogenic line Thatcher Lr16 was inoculated with the leaf rust pathogen, race BBB, race TJB or control. **c**. Actin was used as internal standard. The experiments were repeated three times with similar results

resistance to wheat leaf rust (Fan et al. 2009). Other studies in Arabidopsis have demonstrated that *MPK6*, when silenced, results in plants with compromised resistance to different pathogens (Menke et al. 2004). In barley, *MAP3K*, a MAPKKK, contributes to partial resistance to barley leaf rust (Marcel et al. 2007). The nature of MAPK-induced disease resistance is unknown, but both *ATMPK6* and *ATMPK3* in Arabidopsis have been implicated in priming of disease stress responses (Beckers et al. 2009). Additionally, the Arabidopsis MAPKs *ATMPK4* and *ATMPK6* have been implicated in response to abiotic stresses such as low temperature, low humidity, hyperosmolarity, touch and wounding (Ichimura et al. 2000).

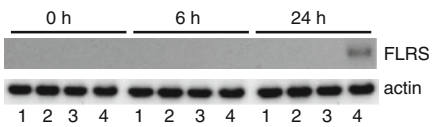


Fig. 3 Transcriptional changes of *TaFLRS* gene in response to Fusarium head blight challenge. 1, Roblin + H_2O; 2, Roblin + FHB; 3, Frontata + H_20; 4, Frontana + FHB. Actin was used as internal standard. The experiments were repeated three times with similar results

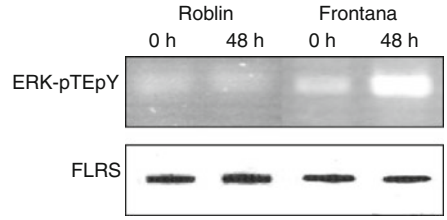


Fig. 4 Immunological detection of TaFLRS after SA treatment of Roblin and Frontana leaf segments using western blot and the antibody against phospho-ERK-type MAPKs. The band in the lower panel represents the leaf proteins immunoprecipitated by a polyclonal antibody again TaFLRS. The upper panel indicates the cross reaction of the immunoprecipitated protein and anti-ERK-pTEpY antibody. This is a representative result from two experiments

Lr16 in wheat belongs to a group of *R* genes that are initiated upon plant-pathogen recognition mediated by a gene-for-gene interaction between a plant *R* gene product and a pathogen *Avr* gene product and the interaction leads to the activation of plant defence responses (Hammond-Kosack and Jones 1996; Jordan et al. 2006; Rudd et al. 2008). In this *P. triticina*-wheat interaction, the rapid *TaFLRS* transcriptional upregulation following the *Lr16*-BBB incompatible interaction was detected in 1.5 h but there was no significant change upon race TJB compatible interaction, suggesting that *TaFLRS* might be involved in the early defence response to leaf rust. Additionally, *TaFLRS* transcription was upregulated in FHB-resistant cultivar Frontana 24 h following inoculation with *F. graminearum* but there was no change in transcript levels in FHB-susceptible Roblin. The significant difference in *TaFLRS* response time between leaf rust challenge and FHB challenge was noticed. It is possible that the slow response to FHB could be due to the low background *TaFLRS* transcript levels in wheat head. Work on other resistant and susceptible cultivars may further evaluate the role of *TaFLRS* in wheat-FHB and wheat-other pathogens interactions. Because *TaFLRS* was upregulated in resistance responses in wheat to both *P. triticina*, an obligate biotroph (Panstruga 2003) and *F. graminearum*, a necrotroph (Jansen et al. 2005; Walter et al. 2010), *TaFLRS* is likely acting downstream to *R* gene and other pathogen receptors, and is involved in generalized defence responses to plant pathogens. When a similar leaf rust pathosystem was examined by microarray analysis, a wheat MAPKK (TaLr1106-B01R) was induced at transcriptional level (Fofana et al. 2007). It would be interesting to see if

this MAPKK interacts with TaFLRS by an in vitro protein interaction analysis (Xing et al. 2008).

In the R/Avr interactions, rapid immune responses such as the HR at the infection site are often mediated by SA, and basal resistance that is triggered by many virulent pathogens also involves SA (Dangl and Jones 2001; Grant and Lamb 2006). Initially, we considered that the *Lr16*/*P. triticina* incompatible interaction might reflect the involvement of SA in *TaFLRS*-mediated plant defence. However, we determined that transcript levels of *TaFLRS* did not change upon SA treatment. We therefore considered that the *TaFLRS* MAPK cascade is regulated at multiple levels including transcriptional, translational and post-translational levels (Xing et al. 2002). TaFLRS was post-translationally activated by dual phosphorylation of T and Y residues in cultivar Frontana during SA treatment but not post-translationally activated in Roblin. This suggests that TaFLRS in resistant wheat cultivars could be involved in the SA signalling pathway by SA-triggered phosphorylation. A tobacco SIPK kinase is similarly regulated at the post-translational level by dual phosphorylation of T and Y residues in the conserved TEY motif (Zhang and Klessig 1997). Activation of *ATMPK4* and *ATMPK6* has been associated with tyrosine phosphorylation rather than with levels of gene transcription or mRNA translation (Ichimura et al. 2000). Also, in the examination of proteins that were phosphorylated in Arabidopsis upon treatment with flagellin, a bacterial elicitor, only a few of the phosphoproteins were found to be regulated at the transcriptional level (Peck 2003). Recently, the wheat MAPKs (TaMPK3 and TaMPK6) were also found to be regulated at post-translational level by dual phosphorylation on T and Y residues (Rudd et al. 2008). Therefore, the emerging picture of regulation of MAP kinases appears to be post-translational, involving T and Y residues.

In summary, a *TaFLRS* has been implicated in wheat-leaf rust and wheat-FHB interactions. It may be possible to manipulate *TaFLRS* expression to attain durable plant disease resistance (Gilbert et al. 2006; Xing et al. 2002). Future studies will elucidate the pathways and function of *TaFLRS* gene in plant growth and development and in cell death regulation so that disease control strategies could be built on an understanding of the pathways and their components in the global biological context.

Acknowledgements This work was supported by Agricultural Bioproducts Innovation Program (ABIP) to D.G., A.L., and T.X., NSERC Discovery Grant to T.X. We thank Dr. Jeannie Gilbert, Dr. Brent McCallum, and Dr. Thérèse Ouellet in Agriculture and Agri-Food Canada for their support.

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