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A type I MADS-box gene is differentially expressed in wheat in response to infection by the stripe rust fungus

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Abstract

The gene, designated *TaMADS2*, was obtained from wheat leaves infected with the wheat stripe rust fungus by *in silico* cloning and RT-PCR. *TaMADS2* encodes a predicted 159-amino-acid polypeptide that contains a highly conserved MADS domain. Phylogenetic analysis revealed that *TaMADS2* is a type I MADS-box gene. The *TaMADS2* transcript was detected in wheat leaves, stems, and roots. The expression of *TaMADS2* was substantially down-regulated in the compatible interaction between wheat and *Puccinia striiformis* f. sp. *tritici* (*Pst*) at 36 and 48 h post-inoculation (hpi) whereas in the incompatible interaction, the down-regulation was only observed at 48 hpi. Exogenous salicylic acid (SA) and abscisic acid (ABA) greatly induced the expression of *TaMADS2* at 12 h post treatment (hpt) whereas methyl jasmonate (MeJA) down-regulated *TaMADS2* at 6 hpt by approximately two-fold.

Additional key words: abscisic acid, methyl jasmonate, *Puccinia striiformis* f. sp. *tritici*, salicylic acid, *TaMADS2*, *Triticum aestivum.*

Introduction

All MADS-box genes encode a strongly conserved MADS domain of approximately 60 amino acid residues which is responsible for DNA binding to CC(A/T)GG boxes in the regulatory regions of their target genes (Zhu and Perry 2005). Plant MADS-box genes can be grouped into two evolutionary lineages, type-I and type-II, and it is proposed that an ancestral duplication before the divergence of plants and animals resulted in these groups (Alvarez-Buylla *et al.* 2000b). In comparison with the type-I genes, type-II genes contain three additional functional regions: a weakly conserved I (intervening) box, a K (keratin-like) box responsible for protein-protein interactions, and a poorly conserved C box as a possible trans-activation domain, and therefore termed the MIKC-type genes (Münster *et al.* 1997). Most MADS-box genes studied belong to type-II genes but type I subfamily has remained largely unexplored (De Bodt *et al.* 2003, Nam *et al.* 2004).

The MADS-box genes, isolated initially as homeotic genes, are among the most extensively studied transcription factor genes in plants (Jack 2001, Ng and Yanofsky 2001). Further studies of floral development led to the formulation of the ABCDE model as a key principle to elucidate the molecular mechanisms of floral organ specification and most of those ABCDE genes encode MADS box proteins (Theissen 2001, Theissen and Saedler 2001). Thus, the central roles of MADS-box genes in flowering have been established and confirmed. Extensive studies also characterized MADS-box genes that are involved in other functions separate from flowering (Alvarez-Buylla *et al.* 2000b, Mao *et al.* 2000) and it is now believed that MADS-box genes encode transcription factors participating in diverse processes of development and growth (Theissen *et al*. 2000,

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Abbreviations: hpi - hour post inoculation; hpt - hour post treatment; HR - hypersensitive response; ORF - open reading frame; qRT-PCR - quantitative reverse transcription polymerase chain reaction.
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Zeng *et al.* 2011). A previous study reported that over-expression of *OsMADS26* induced cell death (Lee *et al.* 2008). However, less it is known about the function of MADS-box genes, especially type-I genes, in the plant-microbe interactions.

Plant defense responses against microbial infection are usually activated by different signals generated during the initial stages of the plant-pathogen interactions (Lamb *et al.* 1989). Transcription factors are believed to play an important role in the transmission of pathogen-derived defense signals to either activate or suppress downstream defense gene expression as well as in the regulation of cross-talk between different signaling pathways (Lorenzo *et al.* 2003). Wheat stripe rust, caused by the obligate biotrophic fungus *Puccinia striiformis* f. sp. *tritici* (*Pst*), often imposes a tremendous threat to the production of wheat worldwide (Chen *et al.* 2002). To elucidate the molecular regulatory mechanism of wheat defense response against *Pst*, we isolated and characterized a type I MADS-box gene, *TaMADS2*, in wheat. Our results indicate that *TaMADS2* was induced by plant hormones and of more particular interest is that *TaMADS2* is differentially expressed during wheat-stripe rust fungus interaction which is the first report of participation of type I MADS-box gene in the plant-microbe interactions.

Materials and methods

Plant cultivation, pathogen inoculation, and phyto-hormone treatment: Wheat (*Triticum aestivum* L.) cv. Suwon 11 and the stripe rust pathogen *Puccinia striiformis* f. sp. *tritici* (*Pst*), strains CYR23 and CYR31, were used throughout this study. Suwon11 is susceptible to CYR31 but resistant to CYR23 by displaying hypersensitive response (HR). Wheat seedlings were grown and maintained as described by Kang *et al*. (2002). Freshly collected urediniospores were inoculated onto the surface of the primary leaves of 7-d-old seedlings with a paintbrush. Control plants were mock-inoculated in the same manner but without spores. After inoculation, all of the plants were kept in the dark for 24 h with 100 % relative humidity and subsequently transferred to a growth chamber set at temperature of 16 °C relative humidity of 85 % and a 16-h photoperiod with irradiance of 125 μmol m^{-2} s^{-1}. Wheat leaves were sampled at 0, 6, 12, 24, 48, 72, and 120 h post inoculation (hpi), quickly frozen in liquid nitrogen, and stored at -80 °C.

For phytohormone treatment, leaves of wheat seedlings (4-week-old) were sprayed separately with 100 μM salicylic acid (SA), 100 μM ethephon (ET), 100 μM methyl jasmonate (MeJA), or 100 μM abscisic acid (ABA) dissolved in 0.1 % (v/v) ethanol. For the mock control, wheat plants were treated with 0.1 % ethanol. The treated wheat leaves were harvested at 0, 2, 6, 12, and 24 h post treatment (hpt). For tissue specificity analysis, leaves, stems, and roots of 4-week-old wheat seedlings were sampled.

Total RNA extraction and reverse transcription: Total RNA of all collected samples was extracted with *Biozol* reagent (*BioFlux*, Tokyo, Japan) according to the manufacturer's instructions. DNaseI treatment was applied to remove genomic DNA. The first strand cDNA was synthesized with *M-MLV* reverse transcriptase kit (*Invitrogen*, Carlsbad, CA, USA) according to the manufacturer's protocol, and Oligo d(T)18 was used as the primer for cDNA synthesis.

Isolation and sequence analysis of *TaMADS2*: *In silico* cloning in combination with RT-PCR method were used to obtain the *TaMADS2* gene. In a previous study (Wang *et al.* 2008), an EST homologous to MADS-box genes was extracted and used as a query probe to search the wheat EST database in GenBank. Homologous wheat ESTs were retrieved and used for *in silico* extension as previously described by Zhang *et al.* (2011). To verify the final assembled sequence, primers FP1 (5'-AACCACCGAGAAAGACGAGAG-3') and RP1 (5'- CTGCTCAAGTCCTTCCTCCCT -3') were used to amplify *TaMADS2* open reading frame (ORF). Total RNA from wheat seedlings was extracted and converted to cDNA which was used as template in PCR for *TaMADS2* amplification. The reaction mixtures were as follows: 2.5 mm^3 of 10 × Taq buffer, 2.0 mm^3 of 25 mM $MgCl_2$, 0.5 mm^3 of 10 mM dNTP, 0.5 mm^3 of 10 μM of each primer, 2 mm^3 of 10 × cDNA, 0.2 mm^3 of 5 U mm^{-3} Taq DNA polymerase, and double distilled water to 25 mm^3. PCR amplification was performed for 35 cycles (95 °C for 30 s, 57 °C for 30 s, 72 °C for 2 min), followed by a final extension step at 72 °C for 10 min. The resulting PCR products were cloned into *pGEM-T* easy vector (*Promega*, Madison, WI, USA) and sequenced with an *ABI PRISM 3130XL* genetic analyzer (*Applied Biosystems*, Foster City, CA, USA). DNA sequences were analyzed with the *DNASTAR* (http://www.dnastar.com), *BLAST* (http://www.ncbi.nlm.nih.gov/blast/), and *ORF Finder* (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) programs. *ClustalW 1.83* and *DNAMAN6.0* (*Lynnon BioSoft*, Vaudreuil, Canada) were used for sequence alignment analyses. *MEGA4* was used for phylogenetic analysis using the neighbor-joining (NJ) method. The *TaMADS2* gene sequence has been deposited in GenBank (GenBank acc. No. JN248615).

Quantitative RT-PCR (qRT-PCR): To analyze the expression levels of *TaMADS2*, relative quantification of gene expression was performed with *SYBR Green*

qRT-PCR mixtures in an *ABI Prism 7500* sequence detection system (*Applied Biosystems*). PCR was performed with the program of 95 °C for 1 min and 42 cycles of 95 °C for 10 s, 57 °C for 20 s, and 72 °C for 40 s. The transcript level of *TaMADS2* was calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with the wheat 18S rRNA gene for normalization. Transcript abundance was assessed with three independent biological replicates. A probability (P) value ≤ 0.05 was used to determine the significance of difference between time-course points, or when relative quantity of RNA was at least two fold higher or lower than that of leaves from control plants. The primers designed for qRT-PCR were: wheat 18S rRNA forward primer: 5'-TTTGACTCAACA CGGGGAAA-3', reverse primer: 5'-CAGACAAATCGC TCCACCAA-3'; *TaMADS2* gene forward primer: 5'- GGTCTCGCTGCTCGTCTT-3', reverse primer: 5'-CTCCATCATTGTTGTTACTT-GC-3'.

Results

To clone *TaMADS2* cDNA, a strategy of a combination of *in silico* cloning and RT-PCR techniques was used. A 893-bp cDNA fragement containing a 480-bp ORF was identified. The ORF of *TaMADS2* encodes a polypeptide

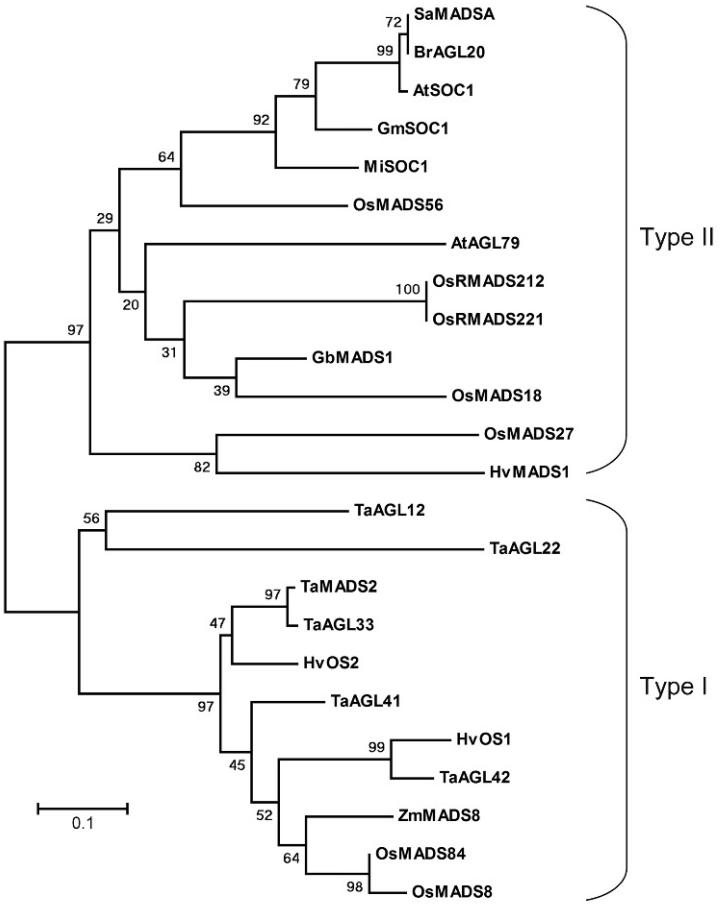

Fig. 1. Phylogenetic analysis of TaMADS2 and related plant MADS-box proteins (GenBank acc. numbers in parenthesis). *Triticum aestivum*, TaMADS2 (JN248615); *Triticum aestivum*, TaAGL33 (ABF57950); *Hordeum vulgare* ssp. *vulgare*, HvOS2 (ADI96238); *Triticum aestivum*, TaAGL41 (ABF57941); *Hordeum vulgare* ssp. *vulgare*, HvOS1 (ADI96237); *Triticum aestivum*, TaAGL42 (ABF57942); *Zea mays*, ZmMADS8 (ACG33795); *Oryza sativa*, OsMADS8 (ABR25605); *Oryza sativa*, OsMADS84 (ABF84007); *Triticum aestivum*, TaAGL12 (ABF57951); *Triticum aestivum*, TaAGL22 (ABF57949); *Mangifera indica*, MiSOC1 (ADX97324); *Arabidopsis thaliana*, AtAGL79 (AAN52802); *Glycine max*, GmSOC1 (ABC75835); *Ginkgo biloba*, GbMADS1 (BAD93165); *Oryza sativa*, OsMADS56 (AAQ23145); *Oryza sativa*, OsRMADS212 (AAS59823); *Oryza sativa*, OsRMADS221 (AAS59832); *Oryza sativa*, OsMADS18 (AAF04972); *Sinapis alba*, SaMADSA (AAB41526); *Brassica rapa* ssp. *chinensis*, BrAGL20 (ABP88100); *Arabidopsis thaliana*, AtSOC1 (NP_182090); *Oryza sativa*, OsMADS27 (BAD29571). The unrooted phylogram was constructed based on NJ analysis. Confidence of groupings was estimated by using 1 000 bootstrap replicates. Numbers next to the branching point indicate the percentage of replicates supporting each branch. The *scale bar* indicates 0.1 change per nucleotide.

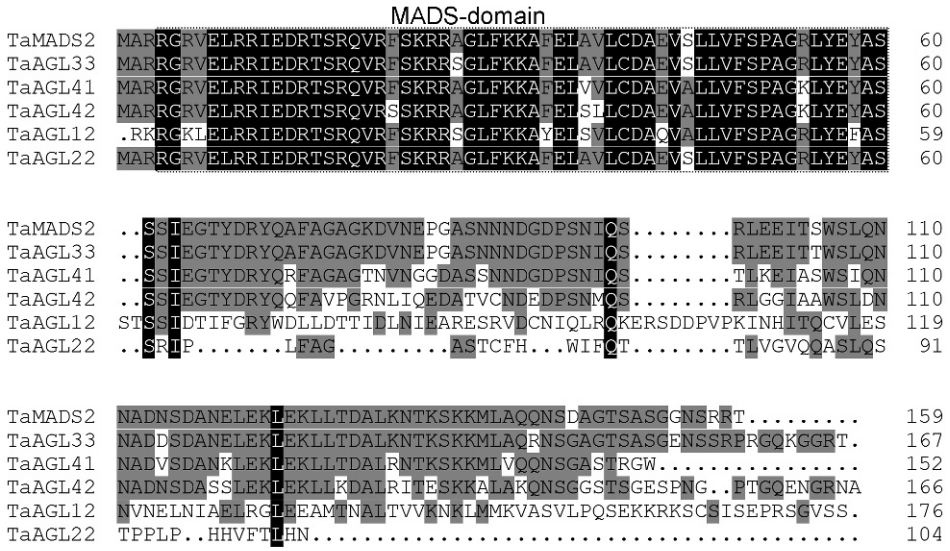

Fig. 2. Alignment of TaMADS2 with other wheat type I MADS-box proteins. The sequences in *dotted box* represent the MADS-domain predicted by the *RPS-BLAST* on *NCBI* website (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

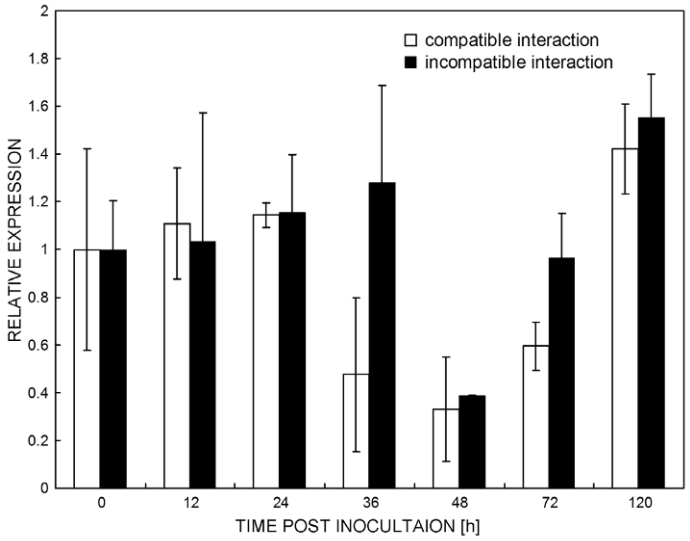

Fig. 3. Expression profiles of *TaMADS2* gene in wheat leaves infected with the stripe rust fungus at different time points. Incompatible interaction: wheat cultivar Suwon11 was inoculated with CYR23. Compatible interaction: wheat cultivar Suwon11 was inoculated with CYR31. Transcript abundance was assessed with three independent biological replicates. *Bars* indicate SD.

of 159 amino acids. The protein sequence was calculated to have a molecular mass of 17.57 kDa and a pI of 9.04. *BLAST* analysis revealed that TaMADS2 shares high similarity with other MADS-box proteins in plants, such as TaAGL33 in wheat (96 % identity and 98 % similarity), HvOS2 in barley (91 % identity and 97 % similarity), and ZmMADS8 in maize (75 % identity and 86 % similarity). Phylogenetic analysis revealed that TaMADS2 is more closely related to type I MADS-box proteins than to type II MADS-box proteins (Fig. 1). Alignment of TaMADS2 with other five wheat MADS-box proteins further indicated that they belonged to type I MADS-box gene without the K domain (Fig. 2). Together, these results strongly support the notion that *TaMADS2* is a type-I MADS-box gene.

To investigate whether the expression of *TaMADS2* gene was tissue-specific, its relative expression in leaves, stems, and roots were determined. qRT-PCR analysis showed that the transcripts of *TaMADS2* were clearly detectable in all these tissues with the highest amount in leaves (about 1.2-fold more than in roots) and the lowest in stems (about 0.6-fold compared with roots).

Using qRT-PCR analysis, we also tested whether the expression of *TaMADS2* in wheat leaves was induced by exogenous applications of phytohormones, such as ABA, ET, MeJA, and SA (Table 1). When treated with ABA, expression of *TaMADS2* was up-regulated between 2 and 24 hpt and peaked at 12 hpt when it was nearly 3.8-fold

Table 1. Expression profiles of *TaMADS2* in wheat leaves sprayed with 100 μM salicylic acid (SA), 100 μM ethephon (ET), 100 μM methyl jasmonate (MeJA), or 100 μM abscisic acid (ABA). Data represent means ± SD of three biological replicates.

Time post treatment [h]	SA	MeJA	ET	ABA
0	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.0	1.0 ± 0.0
2	0.7 ± 0.4	0.8 ± 0.1	1.1 ± 0.2	1.5 ± 0.1
6	0.8 ± 0.0	0.3 ± 0.1	0.8 ± 0.5	1.2 ± 0.2
12	3.7 ± 0.7	0.7 ± 0.2	1.6 ± 0.2	3.8 ± 0.4
24	2.0 ± 0.3	0.6 ± 0.0	1.2 ± 0.1	1.6 ± 0.1

higher than that in the control (0 hpt). After treatment with SA, the *TaMADS2* transcripts kept a relatively steady level at 2 and 6 hpt and then increased sharply at 12 hpt. However, after treatment with MeJA, expression of *TaMADS2* was down-regulated between 2 and 24 hpt with the lowest expression at 6 hpt when it was 3.2-fold lower than that in the control. No significant difference was observed in *TaMADS2* expression between ET treatment and the control at 2, 6, 12, and 24 hpt, respectively.

Quantitative RT-PCR showed that in an incompatible interaction, expression of *TaMADS2* decreased only at 48 hpi (Fig. 3). The expression at 48 hpi was 2.6-fold lower than that in the control. In a compatible interaction, however, from 36 to 48 hpi, the transcript amount of *TaMADS2* steadily decreased to about 3-fold lower level than in the control. Thus, the transcriptional profiles in the compatible and incompatible interactions were similar except for a transiently decreased expression earlier after infection in the compatible interaction compared with the incompatible interaction.

Discussion

In this study, we obtained a MADS-box gene, *TaMADS2*, from wheat infected with the stripe rust fungus by *in silico* cloning and RT-PCR. *TaMADS2* contains a highly conserved MADS-box domain shared by all other MADS-box proteins. Further analysis showed that *TaMADS2* belongs to a type-I MADS-box gene because it contains no well-defined K box that is characteristic of type-II MADS-box genes in plants (Alvarez-Buylla *et al.* 2000b). Multiple sequence alignment of TaMADS2 and other five wheat type I MADS-box proteins revealed strong sequence conservation in the MADS domain, whereas other regions are not highly conserved, suggesting the importance of MADS-box domain in these genes. In addition, the phylogenetic analysis in the present study showed that *TaMADS2* was clustered in a clade with *TaAGL33* which is also a type I MADS-box gene. This result is in agreement with previous phylogenetic analysis of MADS-box genes in wheat (Zhao *et al.* 2006).

In *Arabidopsis*, type I MADS-box genes were divided into three subfamilies: Mα (25 genes), Mβ (20 genes), and Mγ (16 genes), although AGAMOUS LIKE33 (AGL33; At2g26320) was not assigned to any of these (Parenicova *et al.* 2003). The first *Arabidopsis* type I MADS-box gene to be characterized functionally was *AGL80/FEM111* (Portereiko *et al.* 2006) which belongs to the Mγ subclade. RT-PCR analyses indicated that *AGL80* transcripts were detected in most organs including roots, stems, leaves, flowers, and anthers. In this study, qRT-PCR analysis indicated that the transcripts of *TaMADS2* were detectable in all tissues examined including roots, stems, and leaves. In addition, in the cDNA library of wheat leaves infected by avirulent race CYR23 of *Pst* (Wang *et al.* 2008), only one EST encoding *TaMADS2* was found indicating low expression level. This is consistent with the characteristic very low expression of type I MADS-box gene members.

Almost all MADS-box genes, that have been characterized, are involved in flower development or other developmental processes *(*Alvarez-Buylla *et al.* 2000a*,* Theissen *et al.* 2000, Ng and Yanofsky 2001, Nam *et al.* 2004). However, their involvement in plant-pathogen interactions has not been reported. Among all 1 515 annotated uniseqences from the cDNA library of wheat leaves infected by *Pst* (Wang *et al.* 2008), only one unisequence encoding a wheat MADS-box gene, *i.e.*, *TaMADS2*, has been detected. Therefore, we speculate that *TaMADS2* functions in the interaction between wheat and *Pst*. Interestingly, our qRT-PCR results indicated that the expression of *TaMADS2* was substantially down-regulated in the incompatible interaction between wheat and *Pst* (by 2.6-fold at 48 hpi), and a similar down-regulation was observed earlier in the compatible interaction at 36 hpi. However, at 36 hpi in the incompatible interaction, *TaMADS2* expression was 2.7-fold higher than that in compatible interaction. The application of electron microscopy and cytochemistry promoted our understanding the infection and developmental processes of *Pst* (Kang *et al.* 1994, Wang *et al.* 2007). These studies showed that during the first 24 - 36 h in compatible interaction between wheat and *Pst*, the haustoria of *Pst* are differentiated and within 48 h, secondary infection hyphae are formed which subsequently generate large numbers of secondary haustorial mother cells that produce secondary haustoria continuing to branch and develop into colonies. This phase between 24 and 48 h after infection is crucial for *Pst* colonization of wheat and for subsequent obligate

biotrophic life. In contrast, in the incompatible interaction, hypersensitive cell death was observed 24 hpi but cells undergoing HR still looked intact. At 48 hpi, an increasing number of host cells took up stain and started losing their original shape and with advancing incubation time, the percentage of infection sites with necrotic host cells increased. Hence, we speculate that *TaMADS2* may perform positive and important roles in wheat-*Pst* interaction during relatively early stages of infection. In compatible interaction, wheat rust fungi suppress *TaMADS2* expression to facilitate its colonization on wheat leaves. However, in incompatible interaction, *TaMADS2* was down-regulated after the occurrence of extensive cell death and involved in wheat HR to defense against *Pst* infection (Lee *et al.* 2008).

Previous studies reported the participation of plant hormones in regulating the expression of MADS-box genes. For instance, the application of cytokinins (Xu *et al.* 2004), gibberellins (Bonhomme *et al.* 2000), ethylene (Ando *et al.* 2001), and auxins (Zhu and Perry 2005) can affect the expression of MADS-box genes in some plants. Because especially SA and JA/MeJA have been demonstrated to perform crucial roles in plant defense (Feys and Parker 2000, Kunkel and Brooks 2002, Robert-Seilaniantz *et al.* 2007), we were interested in determining whether *TaMADS2* was inducible by these hormones. Our results showed that SA and ABA induced a high expression of *TaMADS2* whereas treatment with MeJA resulted in a significantly down-regulated expression at an earlier time. SA signaling is often effective against biotrophic pathogens whereas the JA/ET signaling pathway is required for effective resistance to necrotrophic pathogens (Jones *et al.* 2007). Crampton *et al.* (2009) reported that pretreatment of pearl millet with SA conferred resistance to a virulent isolate of rust fungus, *Puccinia substriata*, whereas MeJA did not significantly reduce infection levels. The results suggest that the SA defence pathway is involved in rust resistance. In this study, the expression of *TaMADS2* was induced more effectively by the exogenous SA than by MeJA and ET suggesting that *TaMADS2* may participate in the SA-mediated signaling pathway in the wheat-stripe rust interactions. The role of ABA in plant resistance pathways is more complicated. ABA has been implicated in susceptibility to both biotrophs and necrotrophs (Mauch-Mani and Mauch 2005). Therefore, it seems that this hormone acts as a negative regulator of plant defense. However, ABA has also been associated with disease resistance. For example, Dunn *et al.* (1990) demonstrated increased content of ABA in French bean in relation with increased resistance to *Colletotrichum lindemuthianum* when the pathogen was in its progressive biotrophic phase. The role of ABA in disease resistance remains complex owing to its multifaceted function in different tissues and developmental stages of the plant. Recent studies have indicated the role of ABA in disease resistance depends on the type of pathogen, its specific way of entering the host, the timing of the defense response, and the type of affected plant tissue (Ton *et al.* 2009). Maybe *TaMADS2* is also involved in the ABA-mediated signaling pathway in the wheat-stripe rust interactions but its role remains unclear and must be further investigated.

In conclusion, the expression of *TaMADS2*, a type I MADS-box gene isolated from wheat, was regulated differentially upon infection by *Pst* and treatment by exogenous phytohormones. The cloning and characterization of *TaMADS2* gene will enable us to investigate its role in wheat-stripe rust interactions at the molecular level.

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