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Isolation and analysis of *ZmPto* from maize, a homologue to *Pto*

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Abstract

Pto has been reported to play important roles in plant disease responses. The cloning and characterization of the *Pto*-like gene named as *ZmPto* from maize was reported in this research. The open reading frame sequence of *ZmPto* was obtained by using RT-PCR. Sequence analysis showed that *ZmPto* encodes a polypeptide of 405 amino acids with predicted molecular mass of 45.0 kDa and pI of 6.01. Yeast two-hybrid analysis showed the interaction of ZmPto with ZmPti1. RT-PCR analysis indicated that the *ZmPto* expression is induced by salicylic acid (SA), abscisic acid (ABA), mannitol and salt suggesting that *ZmPto* may play important roles in both biotic and abiotic signal transduction pathway. Additionally, the *ZmPto* displays different expression patterns in different tissues indicating its multiple roles in plant.

Keywords: *Zea mays* L., *ZmPti1*, Abiotic stress, Salicylic acid, Yeast two-hybrid.
Abbreviations: Pti_Pto-interacting protein; ABA_abscisic acid; SA_salicylic acid; Avr_avirulence; HR_hypersensitive response; STK_serine/threonine protein kinase; NBS_nucleotide-binding site; LRR_leucine-rich repeat; RT-PCR_reverse transcription-polymerase chain reaction; ORF_open reading frame.

Introduction

Plants are continuously confronted with different potential pathogens. During the long period of fighting against these pathogens, plants have evolved special defense mechanisms. An important form of disease resistance known as gene-for-gene resistance depends on the pathogen delivering a specific ‘‘avirulence signal,’’ generated by the expression of an avirulence (*Avr*) gene, and on the plant perceiving and responding to that signal, an ability conferred by a corresponding resistance (*R*) gene (Dangl, 1994; Keen, 1990). The direct or indirect interactions between the plant R proteins and the pathogen Avr proteins trigger a cascade of defense responses including hypersensitive response (HR) and localized cell death at the site of pathogen ingress halting pathogen spread (Chisholm et al., 2006). In recent years, large numbers of plant *R* genes conferring resistance to viral, fungal and bacterial pathogens have been cloned from a variety of plants including major crop species such as wheat (*Triticum aestivum*), rice (*Oryza sativa*) and maize (*Zea mays*) (Liu et al., 2007; Martin et al., 2003；Dubey and Chandel, 2010). Based on the conserved domains in the R protein sequences, the R protein can be classified into eight classes (Hammond-Kosack and Parker, 2003). Among these classes the NBS-LRR proteins are the most prevalent class. The NBS-LRR proteins have a variable N-terminal domain of approximately 200 amino acids, connected by a predicted NBS domain of approximately 300 amino acids and a more variable tandem array of approximately 10 to 40 short LRR motifs (Jones and Jones, 1997; Traut, 1994). The LRR domains are thought to mediate recognition specificity through a direct or indirect interaction with the Avr protein (Dangl and Jones, 2001; Kumar GM et al., 2009).The tomato *Pto* gene, the first cloned plant *R* gene, belongs to another R gene family encoding a cytoplasmic serine/threonine protein kinase (STK). The Pto protein can interact with the avirulence proteins avrPto and avrPtoB from *Pseudomonas syringae* pv *tomato* resulting in the HR-mediated resistance (Kim et al., 2002; Martin et al., 1993). Over-expression of Pto gene in tomato results in a broad-spectrum resistance, not only to *Pseudomonas* strains but also to many other bacterial and fungal pathogens (Tang et al., 1999). In the Pto screening, Pto-interacting (Pti) proteins are identified as yeast-two hybrid interactors of Pto protein. Pti1, also a STK sharing sequence similarity with Pto, can interact with Pto and is specifically phosphorylated *in vitro* by Pto. The interaction of Pto-Pti1 can cause hypersensitive response to infection indicating the important roles of Pto/Pti1 in disease resistance (Zhou et al., 1995). Herrmann et al. (2006) have identified and analyzed four Pti1-like kinases from maize (ZmPti1a, -b, -c, -d). These kinase genes show tissue-specific expression and their corresponding proteins are targeted to different cellular compartments indicating the multiple functions of Pti1s . Also, in previous study, we have cloned the *Pti*-like kinase gene from maize named *ZmPti1*. ZmPti1 has a kinase activity *in vitro* and

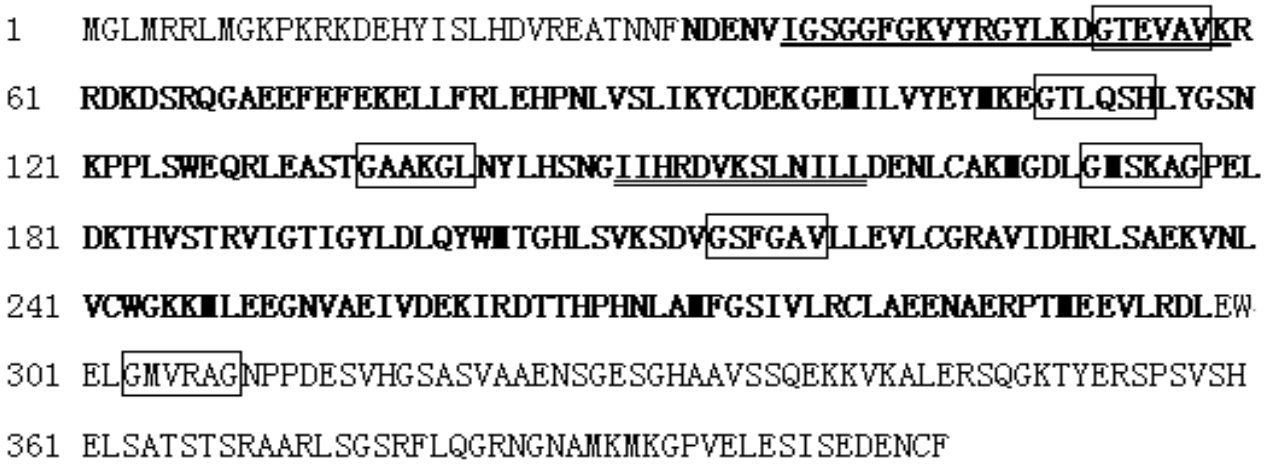

Fig 1. ZmPto amino acid sequence deduced from cloned *ZmPto*; Protein kinase domain is shown in bold type. The underlined amino acids are protein kinase ATP-binding region. The double underlined amino acids are Ser/Thr protein kinases active-site signature. Six sequences in frames are potential N-myristoylation sites

can be induced by SA, low-temperature, mannitol and salt, furthermore, over-expression of *ZmPti1* in *Arabidopsis* leads to an enhanced salt resistance (Zou et al., 2006; Zou et al., 2010). Based on this knowledge, we hypothesized that maybe the Pto/Pti1 in maize can play important roles in both biotic and abiotic stresses. So it is necessary to firstly understand the exact roles of *Pto* gene from maize. In this study, we report the cloning and characterization of *ZmPto* (GenBank Accession no. EU375843), the *Pto*-like gene from maize. The expression pattern of *ZmPto* in various maize tissues and under various treatments was studied. Meanwhile, a yeast two-hybrid analysis was conducted to verify the interaction of ZmPto and ZmPti1 cloned before in our lab. To our knowledge, it is the first report of maize *Pto* gene and this will give us more valuable hints for further study.

Materials and methods

Plant material, growth conditions and experimental treatments

Seeds of maize (*Zea mays* L.) Jingyu7 were surface-sterilized for 5 min in 1% (w/v) sodium hypochlorite, and were washed in distilled water. The following germination, growth conditions and experimental treatments were same as described previously (Zou et al., 2006). The leaves were harvested respectively at 2, 6, 12, 24 and 36 h after various treatments (mannitol, NaCl, ABA and SA). Mature leaves, immature tassels, silks and ovaries were also sampled as described previously (Zou et al., 2006).

Cloning of ZmPto cDNA

The tomato Pto cDNA sequence (accession number U02271) was used as the query probe to search homologues in maize sequence blast search database (http://blast.jcvi.org/er-blast /index.cgi?project=zma1). A highly homologous contig of the maize genome (AZM5_12185) was obtained for analysis of motifs/domain in ScanProsite in PlantsP database (Gribskov et al., 2001). According to the contig sequence, two primers, 5’-TAGACATTTCACATCACCATTG-3’ (forward primer) and 5’-TCAACCTGAGCAGAGCCC-3’ (reverse primer; synthesized at Sunbiotech, China) were used to amplify cDNA encoding ZmPto by reverse transcription-polymerase chain reaction (RT-PCR). RNA was isolated using TRIzol Reagent (Invitrogen, USA). The first strand cDNA was obtained by reverse transcription (RT) using M-MLV reverse transcriptase (Promega, USA) in a 20 µl reaction volume with RNA prepared from maize leaves treated with 2 mM SA for 12 h. RT reaction was performed according to the manufacturer's protocol. The total volume of PCR reaction was 50 µl, containing 2 µl of the first strand cDNA, 0.4 mM of each primer, 1×PCR reaction buffer, 0.2 mM of dNTP and 2 U of Taq DNA polymerase. The reaction was denatured at 94 °C for 5 min, and then subjected to 35 cycles of 94 °C 30 s, 57 °C 45 s and 72 °C 2 min, plus a final extension at 72 °C for 8 min. The RT-PCR product was cloned into pGEM-T vector (Promega) and sequenced (Sangon, China).

RT-PCR analysis

Semi-quantitatively RT-PCR was used to determine the expression level of the *ZmPto* gene. Five micrograms of RNA from different sample was reverse-transcribed into cDNA as described above. The specific primers were designed according to the cDNA sequence. The sense primer was 5’-GGGTTTGGGAAGGTTTATCG-3’ and the antisense primer was 5’-ATCACTGCCCTGCCACATAG-3’. The expected length of the amplified fragment was 566 bp. The total volume of PCR reaction was 25 µl, containing 1 µl of the first strand cDNA, 0.4 mM of each primer, 1×PCR reaction buffer, 0.2 mM of dNTP and 1 U of Taq DNA polymerase. The amplification protocol was 3 min at 94°C (once); 30 s at 94°C, 40 s at 56 °C and 1 min at 72°C (31 times); finally 5 min at 72°C (once). The PCR products were separated on 1% agarose gel and quantified using the higher performance ultraviolet transilluminator (GDS-8000, Gel Documentation System, UVP, USA). Maize *18S rRNA*, amplified with primers 5’-CCATAAACGATGCCGA-3’and 5’-CACCACCCATAGA-ATCAAGA-3’, was used as the internal standard in the experiment. The amplifying procedure was same as amplifying of *ZmPto* except for 24 cycles. The experiments were repeated three times with the similar results and one of them was presented.

The yeast two-hybrid system

The plasmids (pEG202, pJG4-5) and the yeast strain EGY48

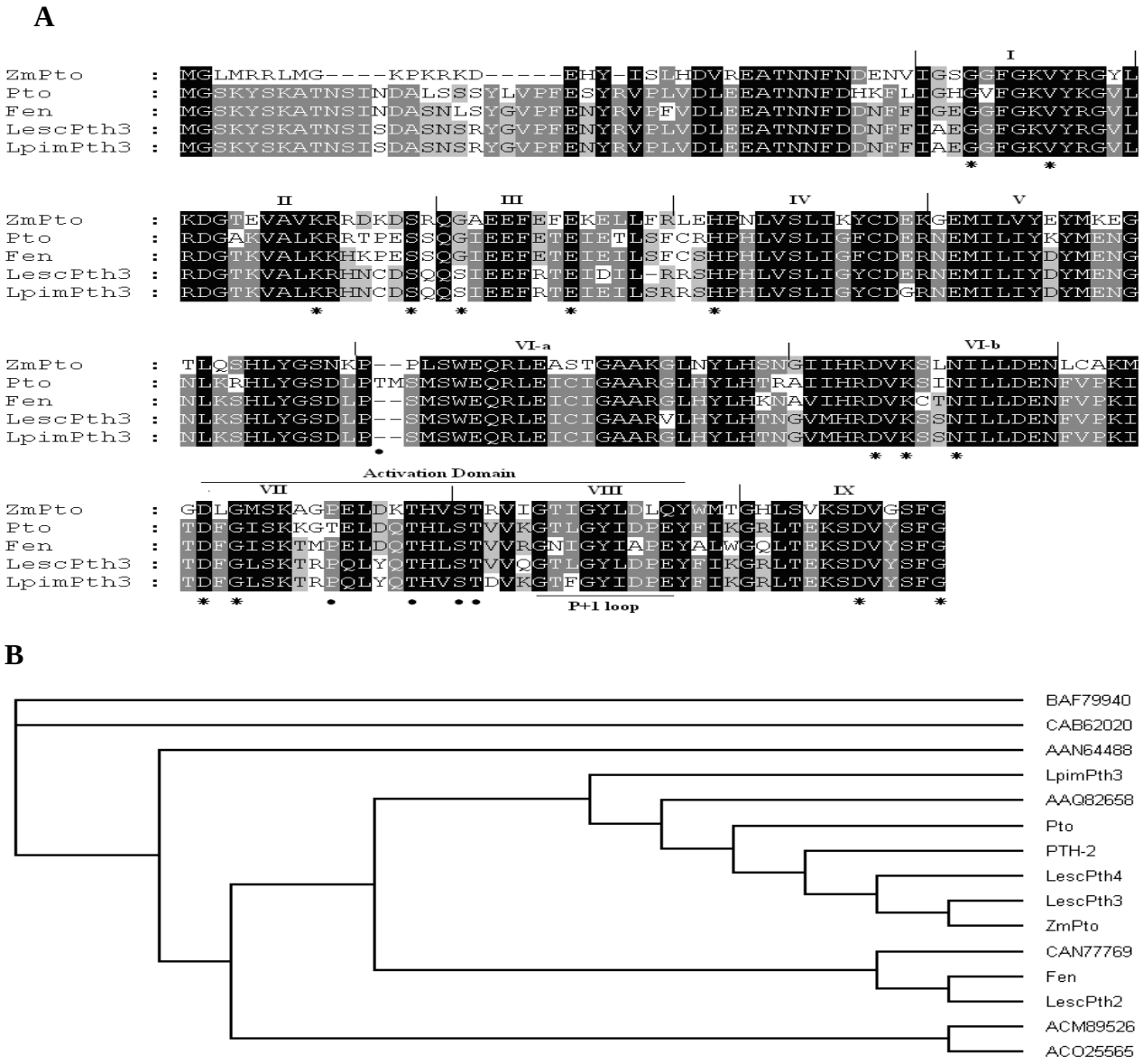

Fig 2. Comparison of the deduced amino acids sequence of ZmPto to those closely related sequences from plant; A, Alignment of amino acid sequence of ZmPto with Pto and Pto-like sequences: Pto (GenBank Accession no. U02271), Fen (GenBank Accession no. Q40126), LescPth3 (GenBank Accession no. AAF76310) and LpimPth3 (GenBank Accession no. AAF76304). The subdomains (labeled with roman numerals), activation domain and P + 1 loop of protein kinases (Hanks et al. 1988; Hanks and Quinn 1991; Vallad et al. 2001) are indicated. Conserved amino acids in plant serine/threonine kinases (Hanks and Quinn 1991) and Pto autophosphorylation sites (Sessa et al. 2000a) are indicated with asterisks and black circles, respectively. Sequences were aligned using ClustalW and GeneDoc. B, Phylogenetic tree based on an alignment of maize ZmPto with those closely sequences from plant in database: putative Pti1 from *Marchantia polymorpha* (GenBank Accession no. BAF79940), *Arabidopsis thaliana* (GenBank Accession no. CAB62020), *Oryza sativa* (GenBank Accession no. AAN64488), *Solanum pimpinellifolium* LpimPth3 (GenBank Accession no. AAF76304), *Capsicum chinense* (GenBank Accession no. AAQ82658), *Cucumis melo* PTH-2 (GenBank Accession no. Q8S519), *Solanum lycopersicum* LescPth2, LescPth3 and LescPth4 (GenBank Accession no.AAF76311, AAF76310 and AAF76309), *Vitis vinifera* (GenBank Accession no.CAN77769), Glycine max (GenBank Accession no. ACM89526), Nicotiana repanda (GenBank Accession no. ACO25565), Pto and Fen.

(*his3*, *trp1*, *ura3*, LexAop-*LEU2*, p8op-*lacZ*), Y864 (*MATa ade2-101 trp1-901 leu2-3*, *112 ura3-52 his3*-D*200 gal4*D *gal80*D *plexAlacZ::URA3*) were provided by Jianping Yang (The Chinese Academy of Agricultural Sciences, China). The basic procedures for the yeast two-hybrid system were conducted as described previously (Yang et al., 2005).

Results

Cloning and sequence analysis of ZmPto

Analysis was made in http://au.expasy.org/tools/dna.html to confirm whether the contig (AZM5_12185) got by blast is a

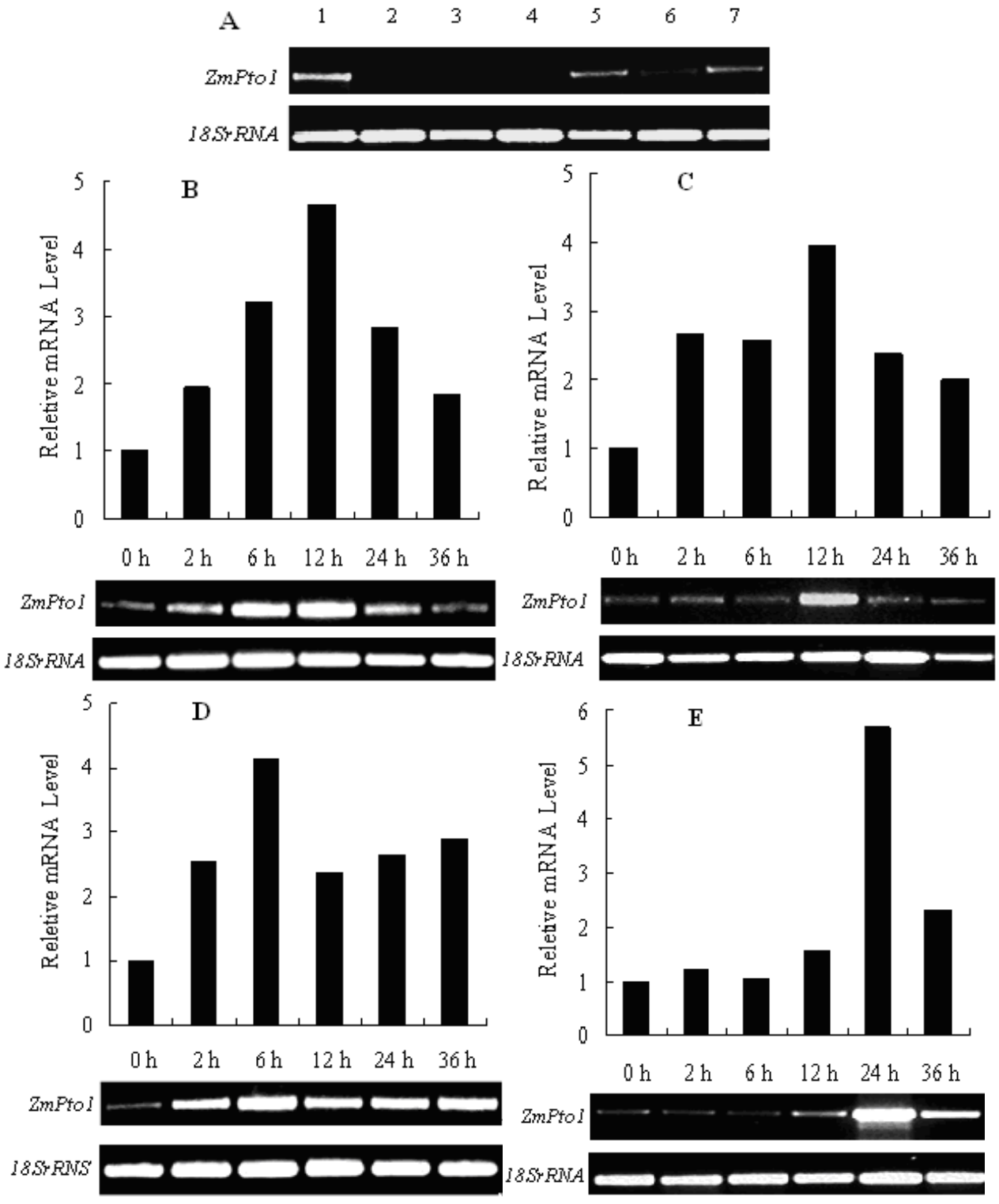

Fig 3. Expression of *ZmPto* in various tissues and in response to various treatments; A, Expression of *ZmPto* in various tissues: 1 roots, 2 coleoptiles, 3 mature leaves, 4 tassels, 5 silks, 6 ovaries, 7 young leaves; B-E, Time-course of *ZmPto* expression in maize young leaves upon SA, ABA, NaCl and mannitol treatment, respectively. The ratio value of the *ZmPto* intensity to the 18S rRNA intensity at 0 h is arbitrarily set to 1 and all the other values are compared to it.

full-length gene. Analysis results showed that there is a in-frame stop codon TGA upstream from the first initiatio codon ATG at the 5'end. And there is an in-frame stop codo TGA at the 3' end (data not shown). These indicate that thi contig comprises a full-length gene. Further blast analysis i NCBI showed that the deduced protein sequence has significant similarity with Pto protein suggesting it's a membe of Pto family. A pair of primers was designed to amplify th open reading frame (ORF) and then the RT-PCR product wa sequenced. Sequencing result showed that the deduced proteir ZmPto, contains 405 amino acids with an estimated molecular mass of 45.0 kDa and an isoelectric point of 6.01. Using the PlantsP program, six potential N-myristoylation sites and a protein kinase ATP-binding region signature IGSGGFGKVYRGYLKDGTEVAVK were found in ZmPto. Additionally, a protein kinase domain (from aa 31st to aa 298th), a protein kinase catalytic domain profile (from aa 31st to aa 301st) and a Ser/Thr protein kinases active-site signature (IIHRDVKSLNILL) were also identified in ZmPto (Fig. 1). These indicate that ZmPto may be a Ser/Thr protein kinase. Multiple sequence alignment of ZmPto protein sequence with other related sequences using the Clustal X program is depicted in Fig.2A. The alignment revealed the conservations of the nine major subdomains (I–IX) which are important for catalytic activity and some invariant amino acids in plant Ser/Thr kinases (Hanks and Quinn, 1991). Also the alignment revealed other features of the Pto protein are conserved in ZmPto such as the activation domain between subdomains VII and VIII, and a P+1 loop site within it, which is responsible for the specific binding of AvrPto (Frederick et al. 1998). In addition, three of the four autophosphorylation sites (Ser or Thr) in the activation domain of Pto (Sessa et al., 2000a) are conserved in the corresponding region of ZmPto. Phylogenetic analysis of the amino acid sequences revealed that ZmPto and *Solanum lycopersicum* LescPth3 are grouped together with closer relationship than with tomato Pto(Fig.2B). These above analyses strongly suggest that the *ZmPto* may encode a functional homologue of Pto.

Expression of ZmPto in various organs and under various treatments

The expression levels of *ZmPto* in different organs: roots, coleoptiles, tassels, silks, ovaries, young and mature leaves were determined by semi-quantitative RT-PCR. The data showed that the transcript levels of *ZmPto* in various organs are significantly diverse. The *ZmPto* are not detected in coleoptiles, mature leaves and tassels; lower in silks, ovaries and young leaves; higher in roots (Fig.3A). The result indicated that *ZmPto* might mainly function in young female reproductive organ, young roots and leaves.To analyze the expression pattern of *ZmPto*, semi-quantitative RT-PCR analysis was also carried out under various treatments: SA, ABA, NaCl and mannitol. The results showed that *ZmPto* expression is up-regulated by SA and its expression level reaches a maximum at 12 h and then declines (Fig.3B). Under ABA treatment, *ZmPto* expression pattern is similar to that of the SA treatment (Fig.3C). Under NaCl treatment, *ZmPto* is highly up-regulated at 2 and 6 h. After 6 hours, *ZmPto* expression is maintained at a stable level (Fig.3D). For mannitol treatment, *ZmPto* is up-regulated significantly at 24 h (Fig.3E). These results indicated that *ZmPto* is up-regulated by all these four treatments.

Interaction of ZmPto with ZmPti1

The high degree of homology shared between ZmPto and Pto,

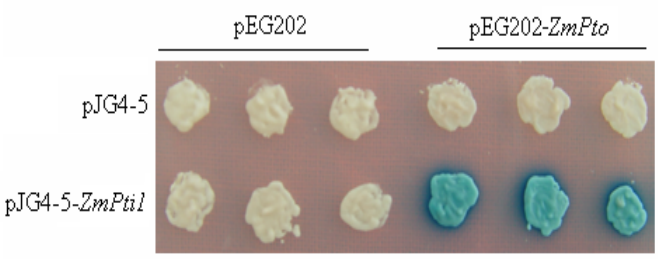

Fig 4. Interaction of ZmPto with ZmPti1; The cDNA of ZmPto was fused into the DNA-binding domain of plasmid pEG202 and transformed into yeast strain EGY48. Otherwise, the cDNA of ZmPti1 was fused into the DNA-activation domain of plasmid pJG4-5 and transformed into yeast strain Y864. The transformed Y864 and EGY48 strains were mated with liquid media lacking histidine, uracil and tryptophan, after which they were grown on SD+Galactose+BU Salts+X-gal medium. The plates were incubated at 30°C for 1 day and photographed. 3 independent, representative colonies are shown for each bait–prey combination.

ZmPti1 and Pti1 as well, suggests that ZmPto and ZmPti1 are functional homologues of Pto and Pti1, respectively. Therefore a yeast two-hybrid assay was used to determine the interaction of ZmPto with ZmPti1. As shown in Figure 4, yeast strains neither carrying empty plasmid nor carrying only one single fusion sequence of ZmPti1 or ZmPto can activate reporter genes. Only the yeast strains carrying both fusion sequence of ZmPti1 and ZmPto can activate reporter genes indicating that ZmPto can interact directly with ZmPti1 in the yeast two-hybrid system.

Discussion

In this study, the ORF of *ZmPto* has been successfully cloned and characterized. Sequence analysis using the PlantsP program showed that *ZmPto* contains one protein kinase domain, one Ser/Thr protein kinases active-site, one protein kinase catalytic domain and one protein kinase ATP-binding region. These indicate that ZmPto might be the Ser/Thr protein kinase. Additionally, six potential N-myristoylation sites were also found in ZmPto protein sequence. N-terminal myristoylation plays a vital role in membrane targeting and signal transduction in plant responses to environmental stress, and this modification is essential for protein function to mediate membrane association or protein–protein interaction (Ishitani et al., 2000; Podelv and Gribsko, 2004). All these above strongly suggest that ZmPto, as the homologue to Pto, may interact with other proteins and response to stress. Furthermore, the following yeast two-hybrid analysis showed that ZmPti1 is one of the candidates which can interact with ZmPto.Previous studies revealed that Pto proteins have nine major conserved subdomains (I–IX) which are important for catalytic activity. Inside tomato Pto protein, the activation domain (between the DFG and APE conserved motifs) contains residues that play key roles in recognition and interaction with other proteins (Sessa et al., 2000a, b). Among these, the most important residues are T204 and Y207 (inside the P + 1 loop) for their being required for the specific recognition and binding of Pto-AvrPto (Frederick et al., 1998). Inside tomato Pto protein, the activation domain also contains four autophosphorylation sites which are required for kinase activity or physical interaction with proteins. Among them, the residue S198 is required for elicitation of the HR (Sessa et al., 2000a). Multiple sequence alignment showed that ZmPto not only has

nine major conserved subdomains but also contains the activation domain and the P+1 loop inside it. Additionally, ZmPto contains the conserved amino acid T204 and Y207 inside the P + 1 loop and all the four autophosphorylation sites inside the activation domain. From multiple sequence alignment we can find that ZmPto also contains the conserved residues V55 and H94 which are important in the interaction with AvrPto, as well as, residues S76 and G79 which are involved in Avr protein binding specificity (Bernal et al., 2005; Scofield et al., 1996). These results strongly suggest that ZmPto might play the similar role to tomato Pto in the signal transduction pathway.Semi-quantitatively RT-PCR analysis showed that the expression of *ZmPto* is up-regulated not only by SA but also by ABA, NaCl and mannitol within the treatment period. This indicates that *ZmPto* may not only play the role in SA-dependent disease defense response pathway but also be responsible for abiotic stress. This result is similar to ZmPti1 we reported before, which partly supports our hypothetical roles of Pto/Pti1 in both biotic and abiotic stresses. In addition, expression in different tissues indicated that *ZmPto* may function in young female reproductive organ similar to that of ZmPti1. This suggests Pto/Pti1 may participate in plant development as well, which was not mentioned in previous work. However, the precise roles of the Pto/Pti1 in stress signal transduction and in plant development need to be further studied.

Acknowledgments

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