Silencing of the Wheat Protein Phosphatase 2A Catalytic Subunit TaPP2Ac Enhances Host Resistance to the Necrotrophic Pathogen Rhizoctonia cerealis

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Eukaryotic type 2A protein phosphatases (protein phosphatase 2A, PP2A) consist of a scaffold subunit A, a regulatory subunit B, and a catalytic subunit C. Little is known about the roles of PP2Ac proteins that are involved in plant responses to necrotrophic fungal pathogens. Sharp eyespot, caused by the necrotrophic fungus Rhizoctonia cerealis, is a destructive disease of wheat (Triticum aestivum), an important staple food crop. Here, we isolated TaPP2Ac-4D from wheat, which encodes a catalytic subunit of the heterotrimeric PP2A, and characterized its properties and role in plant defense response to R. cerealis. Based on the sequence alignment of TaPP2Ac-4D with the draft sequences of wheat chromosomes from the International Wheat Genome Sequencing Consortium (IWGSC), it was found that TaPP2Ac-4D gene is located on the long arm of the wheat chromosome 4D and has two homologs assigned on wheat chromosomes 4A and 4B. Sequence and phylogenetic tree analyses revealed that the TaPP2Ac protein is a typical member of the PP2Ac family and belongs to the subfamily II. TaPP2Ac-4B and TaPP2Ac-4D displayed higher transcriptional levels in the R. cerealis-susceptible wheat cultivar Wenmai 6 than those seen in the resistant wheat line CI12633. The transcriptional levels of TaPP2Ac-4B and TaPP2Ac-4D were significantly elevated in wheat R. cerealis after infection and upon H₂O₂ treatment. Virus-induced gene silencing results revealed that the transcriptional knockdown of TaPP2Ac-4D and TaPP2Ac-4B significantly increased wheat resistance to R. cerealis infection. Meanwhile, the transcriptional levels of certain pathogenesis-related (PR) and reactive oxygen species (ROS)-scavenging enzyme encoding genes were increased in TaPP2Ac-silenced wheat plants. These results suggest that TaPP2Ac-4B and TaPP2Ac-4D negatively regulate defense response to R. cerealis infection possibly through modulation of the expression of certain PR and ROS-scavenging enzyme genes in wheat. This study reveals a novel function of the plant PP2Ac genes in plant immune responses.

Keywords: protein phosphatase 2A, Rhizoctonia cerealis, Triticum aestivum, virus-induced gene silencing, reactive oxygen species
INTRODUCTION

In eukaryotic cells, type 2A protein phosphatases (protein phosphatase 2A, PP2A) are a group of serine/threonine (Ser/Thr) phosphatases. They are regulated by reversible protein phosphorylation and are functionally involved in various biochemical processes (Yu et al., 2005). The PP2A proteins are heterotrimeric holoenzyme complexes and are composed of a scaffold or hook subunit A, a regulatory subunit B, and a conserved catalytic subunit C (Durian et al., 2016). According to the structural characteristics, the regulatory subunits B are further subdivided into B, B′, and B″ (Farkas et al., 2007), all of which are conserved among eukaryotes (Booker and DeLong, 2017). The catalytic subunit C shares high similarity with the catalytic subunits of other Ser/Thr protein phosphatases, such as PP1, PP4, and PP6 (Moorhead et al., 2009). Recent studies indicated PP2A subunits are responsive to reactive oxygen species (ROS) signaling and post-transcriptional levels (Strodtkoetter et al., 2009; Trotta et al., 2017, 2013; Mocek et al., 2013; He et al., 2004). Based on the identity among protein sequences, these PP2Ac proteins are grouped into two subfamilies: AtPP2Ac1, AtPP2Ac2, AtPP2Ac5, LePP2Ac1, and LePP2Ac2 belong to subfamily I, while AtPP2Aa-3, AtPP2Aa-4, LePP2Ac3, LePP2Ac4, and LePP2Ac5 belong to subfamily II (He et al., 2004).

Recent studies indicate that PP2Ac proteins are involved in abiotic stress signaling in several crop species. For instance, in potato (Solanum tuberosum), catalase et al., 2011; Li et al., 2014; Konert et al., 2015a; Rahikainen et al., 2016) showed that in a previous study, catalase was provided by Prof. Jinfeng Yu of Shandong Agricultural University, China. It was significantly upregulated by ABA, and brassinosteroid signal pathways (Garbers et al., 1996; Pernas et al., 2007; País et al., 2009; Tang et al., 2011; Ballesteros et al., 2013; Waadt et al., 2015). For instance, StPP2Ac2 mediated JA signaling and response to wounding stress (País et al., 2009). Recent studies indicated that PP2A subunits were responsive to reactive oxygen species (ROS) signaling and regulated pro-oxidant and antioxidant enzymes at transcriptional and post-transcriptional levels (Strodtkoetter et al., 2009; Trotta et al., 2011; Li et al., 2014; Konert et al., 2015a; Rahikainen et al., 2016). A previous study showed that in a catalase (cat2) pp2a-b'γ double mutant, ROS-induced resistance responses were repressed through the pathways that require PP2a-b'γ activity (Li et al., 2014). However, little is known about the involvement of PP2A in defense responses of an important staple crop, wheat (Triticum aestivum), against fungal pathogens.

Common wheat provides dietary carbohydrates for more than one-third of the population in the world (Zou et al., 2018). Production of wheat is critical for global food security, but its grain yield and quality are negatively affected by various pathogens. Among these, the necrotrophic fungus Rhizoctonia cerealis, which causes wheat sharp eyespot, is one of the most destructive pathogens of wheat around the world (Chen et al., 2008, 2011; Lemańczyk and Kwasa, 2013). After R. cerealis infection, the transport tissues in sheaths and stems of wheat can be destroyed, resulting in blocked transportation of substances that are required for nutrition, lodging, and even dead spikes (Chen et al., 2008). Utilization of resistant wheat cultivars is the most economic and effective strategy to control wheat sharp eyespot. Therefore, molecular breeding is important to uncover the mechanism of wheat defense against R. cerealis and to identify new resistance regulators from the wheat genome. Previous studies in Zhang’s laboratory have identified several genes that are involved in wheat defense response against R. cerealis, such as TaPE1 (Zhu et al., 2014), TaAGC1 (Zhu et al., 2015), TaCAD12 (Rong et al., 2016), and TaRCR1 (Zhu et al., 2017). However, no study about the role of PP2A in wheat defense responses to pathogens has been reported yet.

In this study, we cloned a wheat catalytic subunit of the heterotrimeric PP2A gene named as TaPP2Ac-4D and investigated its functional role in defense response against R. cerealis. TaPP2Ac-4D is located on wheat chromosome 4D and has two homologs that are located on wheat chromosomes 4A and 4B, respectively. The transcriptional levels of TaPP2Ac-4B and TaPP2Ac-4D were upregulated after R. cerealis infection and exogenous H2O2 treatment. Most importantly, silencing the enhanced resistance of TaPP2Ac-4B and TaPP2Ac-4D to R. cerealis infection upregulated the transcriptional levels of PR2 and ROS-scavenging genes (TaCAT1 and TaAPX2) in wheat. This study reveals a novel function of plant PP2Ac genes in the regulation of resistance responses to fungal pathogens.

MATERIALS AND METHODS

Plant and Fungal Materials and Treatments

Three wheat lines/cultivars, including sharp eyespot-resistant CI12633 and susceptible Wenmai 6 and Yangmai 16, were used in this research. The pathogenic R. cerealis strain, WK207, which possesses high-virulence and is prevalent in North China, was provided by Prof. Jinfeng Yu of Shandong Agricultural University, China. All plants of wheat cultivars Yangmai 16, CI12633, and Wenmai 6 were grown in field plots or in a greenhouse under 14-h-light (22°C)/10-h-dark (12°C) conditions (Zhu et al., 2017).
Wenmai 6 were inoculated with *R. cerealis* strain WK207 using the toothpick inoculation method (see below). Samples were collected from stems of wheat plants at 1, 2, 4, 7, 14, and 21 days post inoculation (dpi) with *R. cerealis*. Also, wheat plants treated by toothpick without *R. cerealis* mycelia at each time point were used as mock-inoculation. For expression analysis of TaPP2Ac response to H$_2$O$_2$ treatment, seedlings at the three-leaf stage of Yangmai 16 plants were sprayed with 0.1% Tween-20 solution containing 10 mM H$_2$O$_2$. The plants sprayed only with water containing 0.1% Tween-20 served as a control. Samples (leaves from five plants were quickly cut and pooled as one sample) were collected at 0, 0.5, 1, 3, 6, and 12 h post treatment (hpt) and were stored at -80°C.

**DNA and RNA Extractions**

Genomic DNA for each sample was extracted from 0.1 g of wheat leaves using the CTAB method described by Saghai-Marooof et al. (1984).

To analyze the tissue specific expression of TaPP2Ac, samples (0.1 g for each) were harvested from sheaths, stems, leaves, and spikes and were used for RNA extraction. For expression profile analysis of TaPP2Ac, ROS-related and PR genes, total RNA was extracted from 0.1 g of wheat stems using TRIzol reagent (Invitrogen) and was purified with RNase-free DNase I profile analysis of TaPP2Ac and spikes and were used for RNA extraction. For expression analysis of the target genes were calculated using the 2$-^{ΔΔCT}$ method (Livak and Schmittgen, 2001). The wheat Actin gene, TaActin (GenBank accession number BE425627), was used as an internal reference. The specificity of qRT-PCR products was determined according to the melting curve. The amplification efficiency of qRT-PCR primers were calculated. Three biological replications were performed. Student t-test and Dunnett's test were used to determine the significant differences between controls and treatments.

**TaPP2Ac Gene Silencing in Two Wheat Lines by VIGS**

Barley stripe mosaic virus (BSMV)-based virus-induced gene silencing (VIGS) technique is a fast and effective reverse genetic tool for researching gene functions (Scofield et al., 2005; Zhou et al., 2007; Zhu et al., 2014, 2015, 2017). To generate the BSMV:TaPP2Ac recombinant construct, a 256 bp sequence of TaPP2Ac-4D (from 732 to 987 nucleotides in TaPP2Ac-4D cDNA sequence) was amplified using the primers TaPP2Ac-γ-F and TaPP2Ac-γ-R (Supplementary Table S1) and was sub-cloned in an antisense orientation into the Nhe I restriction site of the RNA$i_y$ of BSMV. The Si-Fi software (Nowara et al., 2010) was used for off-target prediction for the VIGS construct. Capped *in vitro* transcripts were prepared from linearized plasmids containing the tripartite BSMV genome using the RibomAX™ Large Scale RNA Production System-T7 kit (Promega) and the Ribo m$^7$G Cap Analog (Promega) according to the manufacturer's instructions. The resistant wheat line CI12633 and susceptible wheat cultivar Yangmai 16 were, respectively, used as host to perform the VIGS. At the three-leaf stage, the third fully expanded leaves of wheat plants were inoculated with BSMV:TaPP2Ac or BSMV:GFP (as a control) by gently sliding pinched fingers from the leaf base to the tip for a total of six times. After inoculation for 48 h in a humid environment, seedlings were transferred to a greenhouse under 14-h-light (22°C)/10-h-dark (12°C) conditions. After 10 dpi with BSMV, the fourth leaves were collected to monitor BSMV infection based on the transcription of the BSMV cp gene. After 3 days of inoculation with *R. cerealis*, the stems were collected to evaluate the transcriptional levels of TaPP2Ac, ROS-related, and PR genes. The experiment was repeated three times. For one repeat, at least 20 plants were infected by BSMV:GFP and BSMV:TaPP2Ac, respectively. After the experiment, all the BSMV-infected wheat plants were burned down, and the seeds were stored in the refrigerator (−20°C).

**Inoculation With *R. cerealis* and Scoring Response of Wheat Plants**

According to Zhu et al. (2017), two methods including wheat kernels inoculation method and toothpick inoculation method

1http://plants.ensembl.org/Triticum_aestivum/Info/Index?db=core
2http://smart.embl-heidelberg.de/
3http://www.snowformatics.com/si-fi.html
were used to inoculate wheat plants. For wheat kernels inoculation method, at the tillering growth stage, CI12633, Yangmai 16, and Wenmai 6 wheat plants were inoculated on each base-stem with 8∼10 sterile wheat kernels harboring the *R. cerealis* isolate WK207 mycelia. For the toothpick inoculation method, at 20 days after BSMV infection, each stem of the BSMV-infected plants was inoculated with one sterile toothpick harboring the *R. cerealis* WK207 mycelia. The *R. cerealis*-infected plants were sprayed with water twice a day during the first week and then once a day until final disease recording. The infection types (ITs) of wheat plants for each line/cultivar were evaluated at 21 dpi with *R. cerealis*, respectively, based on the disease lesion squares on the base stems. The ITs were categorized from 0 to 5 (i.e., “IT:0” = no lesion, “IT:1” = the lesion appeared on the sheaths rather than stems, “IT:2” = the width of the lesion <25% of the infected stem perimeter, “IT:3” = the width of the lesion >25 and <50% of the infected stem perimeter, “IT: 4” = the width of the disease lesion more than 50% of the infected stem perimeter, “IT: 5” = white spike or dead plant).

**Statistical Analysis**

To determine the significant differences between controls and treatments or between time-course points, Microsoft Excel was used to calculate mean values and standard deviation and to perform Student t-test. The SPSS 19.0 statistical software was used to perform Dunnett’s test.

**RESULTS**

**Identification and Sequence Characterization of TaPP2Ac in Common Wheat**

Given that PP2Ac plays important roles in disease resistance in *Nicotiana benthamiana* (He et al., 2004), we were interested in elucidating the defense roles of PP2Ac in common wheat. Firstly, we cloned the homologous cDNA sequence of the TaPP2Ac-4D from the wheat lines/cultivars CI12633, Yangmai 16, and Wenmai 6. The cDNA sequence of TaPP2Ac-4D from the resistance wheat line CI12633 has been deposited in GenBank (accession number MG461318) with 987-bp length. Nucleic acid sequence analysis showed that no polymorphism exists between the cloned cDNA sequences from these three lines/cultivars. Common wheat is a hexaploid species with A, B, and D subgenomes. To explore the copy number and the chromosomal location of the TaPP2Ac gene, the gene sequence was aligned with the wheat cultivar Chinese spring (CS) genome sequence released in Ensembl Plants (see text footnote 1). This TaPP2Ac cDNA sequence hit three homologous genes that were located on chromosomes 4AS, 4BL, and 4DL, respectively. They were designated as TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D. TaPP2Ac-4A and TaPP2Ac-4B had three transcripts, whereas TaPP2Ac-4D had two transcripts (Figure 1A). All copies of all TaPP2Ac genes possessed 9 to 11 exons based on annotation (Figure 1A). The major differences among the TaPP2Ac homologs were present in the 3’ end sequences (Figure 1A).
Alignments indicated that the amino acid sequences of different copies of TaPP2Ac shared 54.10 to 100.00% identities (Supplementary Table S2). Sequence analysis showed that the predicted ORFs of TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D encoded proteins that are composed of 426, 306, and 313 amino acids, respectively. A phylogenetic analysis of TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D with other PP2A proteins from wheat, Arabidopsis, rice, tobacco, and tomato revealed that TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D clustered with OsPP2A-2, OsPP2A-4, OsPP2A-5, NtPP2A, AtPP2A-3, AtPP2A-4, TaPP2Ac-5A1, TaPP2Ac-5A2, TaPP2Ac-5B1, TaPP2Ac-5B2, TaPP2Ac-5D1, and TaPP2Ac-5D2, all of which are members of subfamily II according to the previous classification of PP2A proteins (Figure 1B; He et al., 2004). Multiple amino acid sequence alignment of TaPP2Ac copies with Arabidopsis and rice PP2A proteins showed that all the predicted TaPP2Ac proteins possessed the structure features of PP2A protein, including a Ser/Thr specific protein phosphatase signature, a tyrosine kinase phosphorylation site, a protein kinase C phosphorylation site, a casein kinase II phosphorylation site, an okadaic acid binding site, and a PP2A activity (Yu et al., 2005; Paix et al., 2010; Figure 2). These results suggest that TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D are members of the PP2A protein in common wheat.

TaPP2Ac-4B and TaPP2Ac-4D Are Responsive to R. cerealis Infection

To test the expression profiles of TaPP2Ac, we investigated the expression of TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D in wheat after R. cerealis infection using qRT-PCR. As shown in Figure 3A, TaPP2Ac-4A transcriptional levels in susceptible wheat line Wenmai 6 were significantly reduced at 1–21 dpi except for 2 and 14 dpi; whereas, in resistant wheat line CI12633, the transcriptional levels of TaPP2Ac-4A were significantly reduced at 1 and 2 dpi. For TaPP2Ac-4B and TaPP2Ac-4D, in susceptible wheat line Wenmai 6, the transcriptional levels were upregulated as early as 1 dpi, with two peaks at 1 dpi (TaPP2Ac-4B) and 14 dpi (TaPP2Ac-4D) and then declined, although the levels were always higher than that of the mock-treated plants. However, in the resistant wheat line CI12633, the transcriptional levels of TaPP2Ac-4D were only elevated at 10 dpi and were relatively weaker; the transcriptional levels of TaPP2Ac-4D were downregulated at 1–21 dpi except for 10 dpi (Figure 3A). These data suggested that the expression of TaPP2Ac-4B and TaPP2Ac-4D rather than TaPP2Ac-4A was induced by R. cerealis infection in the susceptible wheat line Wenmai 6. Based on these observations, TaPP2Ac-4B and TaPP2Ac-4D were selected for further VIGS analysis during the response of wheat to R. cerealis infection.

We subsequently analyzed the expression pattern of TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D in different tissues including stem, leaf, spike, and sheath of the wheat cultivar Yangmai 16. As shown in Figure 3B, without R. cerealis infection, TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D were expressed in the highest levels in the leaves; whereas, at 3 dpi with R. cerealis, the transcriptional levels in spikes and/or leaves were more abundant than those in stems and sheaths, which were the main disease-occurring sites.

TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D Are Differentially Induced by H2O2 Treatment

In order to evaluate the possible linkages between immune signaling and H2O2, we investigated the transcription profiles of TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D post treatment of H2O2 for 0.5, 1, 3, 6, and 12 h. As shown in Figure 4, when seedlings were treated with H2O2, the transcriptional levels of TaPP2Ac-4B and TaPP2Ac-4D were significantly elevated from 0.5 to 12 h, with an induction peak at 6 h (approximate 7.11-fold for TaPP2Ac-4B and 6.15-fold for TaPP2Ac-4D). The TaPP2Ac-4A transcriptional level was only upregulated at 6 h in response to H2O2 treatment (Figure 4).

Silencing of TaPP2Ac-4D and TaPP2Ac-4B Increases Wheat Resistance to R. cerealis

To investigate whether TaPP2Ac plays a negative role in wheat defense response to R. cerealis infection, TaPP2Ac transcript was knocked down in the partial resistant wheat line CI12633 or in the moderately susceptible wheat line Yangmai 16 using BSMV-VIGS technique. Firstly, the VIGS construct used (Figure 5A) here was predicted by the Si-Fi software (see text footnote 3) to show more efficient siRNA hits on TaPP2Ac-4D and TaPP2Ac-4B compared with those on TaPP2Ac-4A (Supplementary Table S3), which suggested the efficient silencing of TaPP2Ac-4B and TaPP2Ac-4D. No off-target was predicted for the VIGS construct in wheat as determined by the Si-Fi software (Supplementary Table S3). At 7 dpi with BSMV, chlorotic mosaic phenotype was observed on the fourth leaves in both BSMV-TaPP2Ac-4B and BSMV-GFP-inoculated wheat Yangmai 16 plants (Figure 5B), and the expression of BSMV cp gene was clearly detected (Figure 5C), which indicated that these recombinant BSMV successfully infected wheat Yangmai 16 plants.

To confirm silencing efficiency, the transcript levels of three copies of TaPP2Ac in stems of BSMV-infected wheat Yangmai 16 plants were evaluated by qRT-PCR. As shown in Figure 5D, the transcriptional levels of both TaPP2Ac-4B...
FIGURE 2 | Multiple amino acid sequence alignment of TaPP2Ac with AtPP2A and OsPP2A. The software DANMAN was used to perform the sequence alignment. The serine/threonine specific protein phosphatase signature is marked by asterisks, the tyrosine kinase phosphorylation site is marked by diamonds, the protein kinase C phosphorylation site is marked by squares, the casein kinase II phosphorylation site is marked by triangles, the okadaic acid binding site is marked by spots, and the PP2A activity regulating site is marked by underline.
and TaPP2Ac-4D were markedly reduced in BSMV:TaPP2Ac-infected Yangmai 16 plants compared with the control plants inoculated with BSMV:GFP, whereas the transcriptional level of TaPP2Ac-4A was not downregulated. This was probably because the TaPP2Ac fragment sequence used for VIGS shared high identities with TaPP2Ac-4D (100%) and TaPP2Ac-4B (98.05%) but low identities with TaPP2Ac-4A1 (71.81%) and TaPP2Ac-4A2 (62.84%) (Supplementary Table S4).

Subsequently, to evaluate the defense role of TaPP2Ac, these BSMV-infected Yangmai 16 wheat plants were further inoculated with the R. cerealis isolate WK207. After 21 days with R. cerealis inoculation, symptom of sharp eyespot disease was present on the stems of BSMV:GFP-infected control Yangmai 16 plants but to a lesser extent in BSMV:TaPP2Ac-infected Yangmai 16 plants (Figure 6A). The average ITs of BSMV:TaPP2Ac-infected Yangmai 16 plants ranged from 2.1 to 2.5, which were lower than that of BSMV:GFP-treated Yangmai 16 plants (ranged from 4.0 to 4.5). The disease lesion areas of BSMV:TaPP2Ac-infected Yangmai 16 plants were 0.56–1.53 cm$^2$, whereas the disease lesion area on the stems of BSMV:GFP-infected Yangmai 16 plants were 1.83–3.67 cm$^2$ (Figure 6B). Downregulation of TaPP2Ac in CI12633 wheat plants showed similar results.

FIGURE 3 | Transcription of TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D in Rhizoctonia cerealis-inoculated wheat (Triticum aestivum). (A) Transcription of TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D in the R. cerealis-resistant wheat line CI12633 and susceptible wheat cultivar Wenmai 6 at 1, 2, 4, 7, 10, and 21 days post inoculation (dpi) with R. cerealis. The expression level of TaPP2Ac in wheat plants treated by toothpick without R. cerealis mycelia (mock-inoculation) at each time point is set to 1. Statistically significant differences between R. cerealis treatment and mock at the same time point are derived from the results of three independent replications (t-test: **P < 0.01). (B) Transcription of TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D in stems, leaves, spikes, and sheaths of wheat Yangmai 16 plants with inoculation by R. cerealis or mock treatment by sterile toothpicks without pathogen for 3 days. The transcriptional level of TaPP2Ac in stems with mock treatment was set to 1. Statistically significant differences are derived from the results of three independent replications (Dunnett’s test: *P < 0.05 and **P < 0.01). TaActin was used as the internal control.
TaPP2Ac Silencing Upregulates the Expression of PR2 and ROS-Scavenging Enzyme Genes

To examine whether TaPP2Ac regulates certain defense-associated genes in wheat response to *R. cerealis*, we analyzed the expression levels of several PR genes including *PR1*, *PR2*, *PR4*, *PR5*, *PR10*, and *PR17* in TaPP2Ac-silenced (BSMV:TaPP2Ac-infected) and control (BSMV:GFP-infected) plants (Figure 7; Supplementary Figure S2). As shown in Figure 7, PR2 transcriptional level was higher in TaPP2Ac-silenced plants than that in control plants, suggesting that silencing of *TaPP2Ac* upregulated *PR2* transcription. To determine whether *TaPP2Ac* modulates H$_2$O$_2$ elimination, we examined the transcriptional levels of wheat genes encoding ROS-scavenging enzymes (*CAT1* and *APX2*) in TaPP2Ac-silenced and control wheat plants. As a result, compared with control wheat plants, the transcriptional levels of *CAT1* and *APX2* were higher in TaPP2Ac-silenced plants than in the control plants (Figure 7). The results suggested that *TaPP2Ac* negatively regulated the transcriptional levels of *CAT1* and *APX2* genes.

DISCUSSION

In the current study, we proved that *TaPP2Ac*-4B and *TaPP2Ac*-4D act as negative regulators in the wheat defense response to *R. cerealis* infection. In plants, multiple genes encoding PP2A subunits were reported (Ariño et al., 1993; Kerk et al., 2002; Yu et al., 2005; Pais et al., 2009). For instance, in *Arabidopsis*, five genes encoding PP2Ac were reported (Ariño et al., 1993; Kerk et al., 2002). Since common wheat is a hexaploid species and composed of A, B, and D subgenomes, wheat genes theoretically have three homoeologous members, one per genome. For *TaPP2Ac*, we also found three homoeologous members located on chromosomes 4AS, 4BL, and 4DL. Comparison of the nucleotide sequences among *TaPP2Ac* homologs revealed some differences that mainly exist in the 3' end sequences. These differences were predicted to lead to alternative splicing and affect the amino acid sequences of the resulting proteins. As supporting evidence for the alternative splicing, in common wheat, *TaPP2Ac*-4A and *TaPP2Ac*-4B each had three transcripts (*TaPP2Ac*-4A1, *TaPP2Ac*-4A2, and *TaPP2Ac*-4A3) for *TaPP2Ac*-4A, and *TaPP2Ac*-4B1, *TaPP2Ac*-4B2, and *TaPP2Ac*-4B3 for *TaPP2Ac*-4B), whereas *TaPP2Ac*-4D had only two transcripts (*TaPP2Ac*-4D1 and *TaPP2Ac*-4D2).

A previous study found that in plants, PP2Ac proteins can be grouped into two subfamilies (He et al., 2004). Here, sequence and phylogenetic analyses indicate that TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D proteins are typical members of PP2Ac subfamily II. Early studies have reported that plant PP2Ac proteins were involved in resistance responses to abiotic stresses (Yu et al., 2003, 2005; Pais et al., 2009; Liu et al., 2014; Hu et al., 2017) and bacterial pathogen infection (He et al., 2004). In *Arabidopsis*, *pp2a-c4* and *pp2a-a1* knock-out mutants exhibit enhanced resistance to the virulent pathogen *P. syringae* pv. *tomato* DC3000 (Segonzac et al., 2014). In this study, employing the BSMV-VIGS technique, the transcriptional levels of *TaPP2Ac*-4B and *TaPP2Ac*-4D were downregulated in wheat, whereas *TaPP2Ac*-4A transcription was not decreased. This is probably due to the low identities (62.84–71.81%) between the sequences of *TaPP2Ac*-4A and the fragmental sequence used in the construction of the VIGS vector. More importantly, knockdown of *TaPP2Ac*-4B and *TaPP2Ac*-4D enhanced wheat resistance to *R. cerealis* infection, indicating that TaPP2Ac negatively regulated the wheat defense response to *R. cerealis* infection. To the best of our knowledge, studies on the role of plant PP2Ac proteins in defense responses against necrotrophic pathogens have not been reported. Thus, this study is probably the first report that reveals the negative effect of a plant PP2Ac protein on defense response to necrotrophic fungal pathogens and broadens the understanding of biological functions of PP2Ac proteins in plant species.

By comparing this current report with previous functional studies on PP2Ac proteins, we can find a functional diversity between PP2Ac subfamily I and II in plants (Yu et al., 2003, 2005; He et al., 2004; Pais et al., 2009; Ballesteros et al., 2013; Liu et al., 2014; Hu et al., 2017). In brief, members of the PP2A subfamily I were usually involved in brassinosteroid and ABA signalings, as well as plant responses to salt and hemibiotropic pathogen stresses (He et al., 2004; Pernas et al., 2007; Pais et al., 2009; Tang et al., 2011; Hu et al., 2017), whereas members of the PP2A subfamily II likely play important roles in root development (Ballesteros et al., 2013) and in defense responses against necrotrophic fungal pathogens. The different functions...
FIGURE 5 | Silencing of TaPP2Ac by barley stripe mosaic virus (BSMV)-induced gene silencing in sharp eyespot moderately susceptible wheat Yangmai 16. (A) Scheme of genomic RNAs of BSMV construct and the construct of the recombinant virus expressing the wheat gene TaPP2Ac, BSMV:TaPP2Ac. The orientation of the TaPP2Ac insert is indicated by dark boxes. (B) Mild chlorotic mosaic symptoms observed on leaves at 7 days post inoculated (dpi) with BSMV:GFP or BSMV:TaPP2Ac. (C) RT-PCR analysis of the transcription levels of BSMV coat protein (cp) gene in the wheat plants infected by BSMV:GFP or BSMV:TaPP2Ac. (D) The relative transcript level of TaPP2Ac-4A, TaPP2Ac-4B, and TaPP2Ac-4D in BSMV:TaPP2Ac-infected wheat Yangmai 16 plants is relative to that seen in BSMV:GFP-infected (control) plants. BSMV:TaPP2Ac-1∼3 indicate mean value from three independent experiments. The transcript levels of three TaPP2Ac genes in BSMV:GFP-infected plants from three independent experiments were set to 1. At least 20 plants were infected by BSMV:GFP and BSMV:TaPP2Ac for one repeat. Significant differences were analyzed using Dunnett’s test (*P < 0.05 and **P < 0.01). Deviation bars indicate SD.

may be partially due to the sequence differences. Even though the key residues and domains, such as the okadaic acid binding and PP2A activity regulating sites, are conserved in members of the two subfamilies, an important residue Asp284 that makes four intermolecular hydrogen bonds with the PP2A subunit A (Xing et al., 2006) shows divergence between the two subfamilies. The Asp284 is well conserved in PP2A subfamily II members (such as TaPP2Ac, AtPP2Ac-3, and AtPP2Ac-4) but is substituted with Glu284 in subfamily I members (such as AtPP2Ac-1, AtPP2Ac-2, and AtPP2Ac-5). The sequence variation probably leads to the binding specificity of the two PP2A subfamilies to PP2A subunit A. It will be interesting to further study how the sequence variation affects the functional diversity. Furthermore, in addition to PP2A subunit C encoded by five genes, the subunits A and B were also encoded by multiple genes (3 genes for subunit A, 17 genes for subunit B) in Arabidopsis (Farkas et al., 2007), which could form at least 255 novel forms of PP2A (Hu et al., 2017). This characteristic may be another explanation for the functional diversity of PP2Ac proteins in plants.

Type 2A protein phosphatases, usually acting as regulators, participate in plant immune response (Durian et al., 2016). For example, in Arabidopsis, pp2a-b’γ mutant increased the
abundance and phosphorylation levels of PR proteins including PR1, PR2, and PR5 (Trotta et al., 2011). In this study, TaPP2Ac was also found to have a negative effect on the expression of PR2. Silencing of TaPP2Ac upregulated the expression of PR2. This result was not in accordance with the report of He et al. (2004), according to which silencing of NbNPP4-1 and NbNPP4-2 activated the expression of PR1a, PR1b, and PR5 genes in N. benthamiana (He et al., 2004). TaPP2Ac and NbNPP4 possibly act through different subsets of PR genes in plant defense responses to different life-type pathogens. Alternatively, another explanation is that TaPP2Ac belongs to subfamily II, whereas NbNPP4 is a member of subfamily I of the plant PP2Ac proteins (He et al., 2004). In plants, ROS is an important signaling molecule and is usually involved in signal transduction in numerous pathways (Rahikainen et al., 2016).
During interaction between plants and pathogens, pathogen infection triggers oxidative bursts, which occur at the early stages of pathogen infection, subsequently induce hypersensitive cell death (Tenhaken et al., 1995; Garcia-Brugger et al., 2006). This is usually beneficial for plants as a mechanism to restrict the progression of invading pathogens, including biotrophs and hemibiotrophs (Torres et al., 2006; Lehmann et al., 2015).

*R. cerealis* infection promoted the generation of ROS (Zhu et al., 2015). Catalase (CAT) is a major scavenging enzyme that quenches photorespiratory H$_2$O$_2$ in peroxisomes (Mhamdi et al., 2010). Enzymes that scavenge ROS and antioxidants play important roles in plant defense responses to certain necrotrophic pathogens (Ku´zniak and Skłodowska, 2004; Sharma et al., 2007). Homeostasis of ROS, maintained by ROS-scavenging enzymes (such as CAT and ascorbate peroxidase 2, APX2) and ROS-producing enzyme was found to be crucial for role of TaAGC1 in wheat resistance response to the necrotrophic pathogen *R. cerealis* (Zhu et al., 2015). Segonzac et al. (2014) showed that PP2A led to the flagellin-induced ROS burst into the apoplast. In this study, TaPP2Ac-4D was responsive to H$_2$O$_2$ stimulus, suggesting that TaPP2Ac-4D may be involved in ROS signaling. Furthermore, the transcriptional levels of CAT1 and APX2 were significantly elevated in the TaPP2Ac-silenced wheat plants than in the control wheat plants, indicating that TaPP2Ac negatively regulated the expression of *CAT1* and *APX2*. These results are in line with the finding that the transcript level of *APX2* encoding a H$_2$O$_2$ scavenging enzyme increased in *pp2a-b*/*γζ* double mutants (Konert et al., 2015b); the finding that PP2A-B1 is required to regulate the transcript abundance of genes encoding antioxidant enzymes including mitochondrial AOX1A and 1D and monodehydroascorbate reductase 2 (MDAR2) and chloroplastic copper/zinc SOD 2 (CSD2) (Trotta et al., 2011; Li et al., 2014; Konert et al., 2015a).

In summary, wheat PP2Ac negatively regulated the expression of *PR2*, *CAT*, and *APX2*, leading to repression of host defense response against a necrotrophic pathogen *R. cerealis*. Thus, silencing *TaPP2Ac-4B* and *TaPP2Ac-4D* enhances wheat resistance to *R. cerealis* infection. This study uncovers a novel function of the plant PP2Ac gene in plant immune responses and identifies a potential genetic resource for improving wheat resistance to *R. cerealis* using gene editing system.

**AUTHOR CONTRIBUTIONS**

ZZ and XZ designed the experiment and wrote the manuscript. XZ, YW, ZS, and LL performed the experiments and analyzed the data.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01437/full#supplementary-material

**FIGURE S1 | Silencing of TaPP2Ac transcription enhanced resistance of wheat line CI2633 to *Phytoctiosa cerealis*. (A) qRT-PCR analysis of the transcriptional level of TaPP2Ac in the wheat plants infected by BSMV:GFP (control) or BSMV:TaPP2Ac. (B) Sharp eyespot symptoms of the BSMV:GFP- and BSMV:TaPP2Ac-infected CI2633 plants at 21 dpi with *R. cerealis*. (C) Disease lesion size in BSMV:GFP- and BSMV:TaPP2Ac-infected CI2633 plants at 21 dpi with *R. cerealis*. BSMV:GFP-1 and BSMV:TaPP2Ac-1 indicate mean value of each group from three independent replications. At least 20 plants were infected separately by BSMV:GFP and BSMV:TaPP2Ac for one repeat. Significant difference of BSMV:TaPP2Ac-infected wheat plants relative to the corresponding mean of control for each experiment was analyzed using Student t-test (*P < 0.05 and **P < 0.01).**

**FIGURE S2 | Transcriptional analysis of pathogenesis related genes in wheat Yangmai 16.** The reported transcription levels of the tested gene in the BSMV:TaPP2Ac-infected wheat plants are relative to those in the BSMV:GFP-infected (control) plants. BSMV:TaPP2Ac-1 and BSMV:TaPP2Ac-3 indicate mean value from three independent experiments. The transcription levels of target genes in BSMV:GFP-infected plants from three independent experiments were set to 1. At least 20 plants were infected separately by BSMV:GFP and BSMV:TaPP2Ac for one repeat. Significant differences were analyzed using Dunnett’s test (*P < 0.05 and **P < 0.01). Bars indicate standard deviation of the mean.

**TABLE S1 | Primers used in this study.**

**TABLE S2 |** The identities between amino acid sequences of different copies of TaPP2Ac.

**TABLE S3 |** Prediction of TaPP2Ac off-target transcripts in wheat.

**TABLE S4 |** The identities between the sequences of TaPP2Ac fragment used for VIGS and TaPP2Ac different copies.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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