TaMCA1, a regulator of cell death, is important for the interaction between wheat and Puccinia striiformis

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Metacaspases are present in fungi, protozoa and plants based on predicted structural homologies with the catalytic domains of caspases. In plant systems, metacaspases are subdivided into two types (type I and type II) based on their structures. Specifically, type I metacaspases have an N-terminal pro-domain that is not identified in type II, while type II metacaspases harbor a longer linker region between the putative small (p10) and large (p20) subunits. In the last decade, several metacaspase genes have been found to be involved in cell death. For example, the yeast metacaspase (YCA1) knock-out (yca1Δ) survives in the presence of hydrogen peroxide (H2O2). AtMC1, the homologue of YCA1 in Arabidopsis thaliana, was up-regulated in plants challenged with bacterial pathogens. A recent study reported that AtMC1 and AtMC2 antagonistically control hypersensitive response (HR)-associated cell death that is activated by intracellular immune receptors. Similar to the function of AtMC1, AtMC4 plays a positive regulatory role in biotic and abiotic stress-induced cell death. Interestingly, further study showed that AtMC8 might be involved in the cell death induced by UVC or H2O2, and AtMC8 knockout lines exhibit reduced cell death. TaMCA4 is a novel plant metacaspase gene cloned from wheat (Triticum aestivum). Knockdown of TaMCA4 expression enhances the susceptibility of the host plant to the avirulent P. striiformis f. sp. tritici (Pst) race CYR23 and reduces the necrotic area per infection site. The HR is a rapid plant-initiated cell death that is associated with the recognition of avirulence products by the corresponding resistance genes. Additionally, HR helps plants defend themselves against pathogens by sacrificing plant cells at the infection sites to limit pathogen growth. Stripe rust caused by Pst is one of the most destructive of the fungal wheat diseases worldwide. However, the physiological roles of type I metacaspase genes in the wheat-Pst interaction have not been well characterized. In the present study, we isolated an AtMC1 homolog TaMCA1 in wheat. The TaMCA1 contained typical structural features of type I metacaspases domains and is located in cytosol and mitochondria. TaMCA1 inhibited cell death in tobacco and wheat cells. Quantitative reverse-transcription polymerase chain reaction

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(qRT-PCR) analyses showed that TaMCA1 was up-regulated in wheat leaves challenged by *Pst* race CYR23 and CYR31. Furthermore, knockdown of TaMCA1 in wheat using virus-induced gene silencing (VIGS) enhanced plant disease resistance to *Pst* race CYR31, and TaMCA1 was partly complement the function of the *YCA1*.

**Results**

**Cloning of the TaMCA1 homologue and sequence analyses.** One wheat metacaspase homologue with a complete open reading frame (ORF) was cloned from wheat ‘Suwon11’. The predicted ORF encoded a protein of 292 amino acid residues with a molecular weight of 32.1 kDa. Sequence alignment with the *T. aestivum* cv. Chinese Spring (CS) genome sequence revealed that three copies were located on chromosomes 1A, 1B and 1D. Multi-sequence alignments with other plant metacaspase proteins revealed that the protein contained structural features common to the type I family (Fig. S1), and a phylogenetic tree analysis confirmed its relatedness to *AtMC1* and other plant metacaspase proteins (Fig. S2). Therefore, we named the gene TaMCA1 in this study (KU958719).

TaMCA1 exhibited no caspase-1 activity in vitro. Purified TaMCA1 was assayed with western blotting experiments (Fig. S3a). To measure the activity of TaMCA1 in vitro, the fluorogenic substrate Ac-YVAD-AMC (a substrate of caspase-1) was utilized as previously described. The total protein extracted from the wheat leaves was used as a control. The caspase-1 cleavage product was present in the protein extracted from the wheat leaves, whereas no fragments were detected in the TaMCA1 expressed in *E. coli* strain (Fig. S3b).

Transcriptional changes of TaMCA1 induced by *P. striiformis* f. sp. *tritici* infection. After inoculating seedling ‘Suwon11’ wheat plants with the avirulent race CYR23 or the virulent race CYR31, qRT-PCR was performed to determine the transcript profiles of TaMCA1 during *Pst* infection. In the compatible interaction (CYR31), the expression of TaMCA1 was induced at 24 hours post inoculation (hpi), subsequently peaked at 48 hpi, and gradually reduced at 72 and 120 hpi. In contrast, TaMCA1 was only minimally up-regulated at 72 hpi in the incompatible interaction (CYR23) (Fig. 1).

Subcellular localization of TaMCA1. In plants, several compartments including vacuole, cytosol, chloroplast and mitochondrion display caspase activity. *AtMC1* was cytosolic enzymes, and it was predicted to be localized at mitochondria. To determine the subcellular localization of TaMCA1, the TaMCA1-GFP fusion protein was expressed in wheat seedlings protoplasts, and existed in the form of dots in cytosol. To further confirm its subcellular localization, TaMCA1-GFP was co-expressed with SLO2-DsRed, a well-known mitochondria marker protein (At2g13600). As shown in Fig. 2, the green fluorescence was distributed in cytosol, and a few colocalized with SLO2-DsRed at mitochondria.

**TaMCA1 suppresses cell death in N. enthamiana and T. aestivum.** In the compatible interaction (CYR31), the expression level of TaMCA1 exhibited a significant up-regulation at 48 hpi. Therefore, we speculate that TaMCA1 may play an important role in the cell death to *Pst* in wheat. To prove this idea, TaMCA1 was transiently overexpressed in *N. benthamiana* using potato virus X (PVX) delivery in combination with the Bax system either alone or followed 24 h later with an *A. tumefaciens* strain carrying the mouse Bax gene. The results indicated that the tobacco leaves infiltrated with Bax (Fig. 3a: circle 1), infiltration buffer (BF) + Bax (Fig. 3a: circle 2), empty vector (EV) + Bax (Fig. 3a: circle 7) or eGFP + Bax (Fig. 3a: circle 8) exhibited a cell death phenotype, and green fluorescence could be detected at 3–7 days in the eGFP treatment (Fig. S4), which indicated that the...
Figure 2. Subcellular localization of the TaMCA1 protein. Laser-scanning confocal micrographs showing the expression of fluorescent proteins in wheat seedlings protoplasts. The green channel shows the localization of TaMCA1-GFP; the red channel shows the localization of SLO2-DsRed, a mitochondrial marker protein (At2g13600). Bar = 20 μm.

Figure 3. Transient expression of TaMCA1 in Nicotiana benthamiana. (a) Transient expression of TaMCA1 in the N. benthamiana leaves infiltrated with buffer or A. tumefaciens stains containing a PVX vector carrying the gene (TaMCA1, or Avr1b) or a control gene (eGFP) either alone (circles 1, 3 and 5) or followed 24 h later with A. tumefaciens cells carrying a mouse Bax gene (circles 2, 4, 6, 7 and 8). The photos were taken at 6 d after infiltration. 1, Bax; 2, Buffer → 24 h → Bax; 3, Avr1b; 4, Avr1b → 24 h → Bax; 5, TaMCA1; 6, TaMCA1 → 24 h → Bax; 7, EV → 24 h → Bax; 7, eGFP → 24 h → Bax. (b) TaMCA1 suppressed Bax-mediated programmed cell death in N. benthamiana leaves using double barreled particle bombardment as indicated. The dotted line marks the position of a divider used to prevent the overlap of two bombardment areas. (c) The average numbers of blue spots per shot were observed by light microscopy. EV, pUC empty vector; TaMCA1, pUC-TaMCA1; Bax, pUC-Bax; Gus (β-glucuronidase), pUC-Gus.
work system was operating normally. Simultaneously, the tobacco leaves infiltrated with TaMCA1 (Fig. 3a: circle 5) or Avr1b (Fig. 3a: circle 3) exhibited no differences. However, the tobacco leaves infiltrated with TaMCA1 + Bax (Fig. 3a: circle 6) or Avr1b + Bax (Fig. 3a: circle 4) suppressed cell death, which indicated that TaMCA1 is related to cell death via the suppression of the cell death induced by the mouse Bax gene in tobacco, and this supposition was proven again by the Bio-Rad Gene Gun for co-bombardment assays in N. benthamiana as described18.

Blue spots observed on leaves represented the quantity of living cells, when the tobacco leaves were bombarded with EV + Bax + Gus (β-glucuronidase, Gus), a 79.8% reduction in the number of blue spots was observed compared to leaves that were shot with TaMCA1 + Bax + Gus (Fig. 3b,c). To determine whether TaMCA1 was able to suppress cell death in wheat, we used the attachment of the Bio-Rad Gene Gun for bombardment assays with wheat leaves as described19,20. Numerous blue spots were observed on wheat leaves bombarded with EV + Bax + Gus, EV + Bax + Gus, TaMCA1 + Gus or TaMCA1 + Bax + Gus (Fig. 4a). As shown in Fig. 4b, the number of blue spots on the leaves bombarded with EV + Gus, TaMCA1 + Gus or TaMCA1 + Bax + Gus showed no significant change. However, when the wheat leaves were bombarded with EV + Bax + Gus, a 60% reduction in the number of blue spots was observed compared to leaves that were shot with TaMCA1 + Bax + Gus. Thus, our results showed that TaMCA1 suppressed the cell death triggered by the mouse Bax gene in both N. benthamiana and T. aestivum.

Figure 4. Transient expression of TaMCA1 in T. aestivum. Over-expression of TaMCA1 in T. aestivum leaves using a single barreled particle bombardment. (a) The DNA mixtures used to bombard different groups of leaves are indicated. (b) The average numbers of blue spots per shot were observed by light microscopy. The different letters represent significant differences [P < 0.05 according to analysis of variance (ANOVA)]. EV, pUC empty vector; TaMCA1, pUC-TaMCA1; Bax, pUC-Bax; Gus (β-glucuronidase), pUC-Gus.
Knocking down TaMCA1 increased the resistance of wheat to Pst. Based on the expression profile of TaMCA1 during Pst infection, the barley stripe mosaic virus (BSMV)-based VIGS system was applied to further characterize TaMCA1’s function in the interaction of wheat and Pst. Two pairs of primers were designed specifically to knockdown TaMCA1. Moreover, the silencing of the wheat phytoene desaturase gene (PDS) was used as the positive control for the gene silencing system to confirm whether our VIGS conditions were functioning correctly. The result showed that the plants treated with 1 × Fes buffer, BSMV-γ, BSMV:TaMCA1-1 or BSMV:TaMCA1-2 displayed mild chlorotic mosaic symptoms by 9 dpi but exhibited no obvious defects in further leaf growth (Fig. 5a). The fourth leaves of the wheat plants that were pre-treated with 1 × Fes buffer, BSMV-γ, BSMV:TaMCA1-1 or BSMV:TaMCA1-2 were then inoculated with urediospores of the Pst virulent race CYR31. On average, the knockdown of TaMCA1 expression limited the number of uredium developments, which was equivalent to the development of an increased resistance type to the wheat stripe rust fungus (Fig. 5b). Additionally, the fungal biomass in both TaMCA1-knockdown plants was significantly reduced at 120 hpi compared with the control plants (pre-injected with 1 × Fes buffer or BSMV-γ) (Table S1), which suggested that the wheat stripe rust fungus growth or development were restricted to a certain extent in both TaMCA1-knockdown plants. The transcripts level of TaMCA1 was significantly suppressed to different extents (71–78%) compared with the BSMV-γ-treated plants (Fig. 5c), which indicated that TaMCA1 was silenced in both TaMCA1-knockdown plants.
Histological changes of *Pst* growth and host response. A microscopic examination revealed that there was no obvious difference in the hyphal branches between the control plants and the plants pre-treated with BSMV:TaMCA1-1 or BSMV:TaMCA1-2 at 24 or 48 hpi (Table 1). Moreover, the hyphal length of the wheat pre-treated with BSMV:TaMCA1-1 or BSMV:TaMCA1-2 were significantly (*P* < 0.05) shorter than those observed in the control plants at 48 hpi (Table 1), and the colony size in both TaMCA1-knockdown plants was significantly reduced compared with the sizes observed in the control plants (*P* < 0.05) at 120 hpi (Table 1).

To further understand the correlation between *Pst*-induced TaMCA1 and *Pst*-induced cell death, we assayed the expression levels of a few selected genes in TaMCA1 knockdown plant after infection with the stripe rust fungus, including catalase (TaCAT), class III peroxidase (TaPOD) and superoxide dismutase (TaSOD), *Triticum aestivum* metacaspase 4 (*TaMCA4*) and *Triticum aestivum* defender against cell death (*TaDAD2*). As shown in Fig. 5d, the levels of TaMCA4 and TaDAD2 showed no change in comparison with BSMV:γ-treated plant. But, the transcript levels of TaCAT, TaPOD and TaSOD were down-regulated in TaMCA1-knockdown plant, particularly at 24 hpi. Together, these results suggested that TaMCA1 may be involved in plant ROS accumulation to influence plant resistance.

Enhanced reactive oxygen species accumulation in TaMCA1-knockdown plant. To further confirm the hypothesis that TaMCA1 may be involved in plant ROS accumulation to influence plant resistance, we assayed the production of hydrogen peroxide (H$_2$O$_2$), the most important component of ROS. The results showed that H$_2$O$_2$ accumulation in both TaMCA1-knockdown plants with *Pst* race CYR31 was significantly (*P* < 0.05) greater than that in the control plants with *Pst* race CYR31 at 24 hpi (Table 1), which was consistent with the result in Fig. 5d. These findings suggest that TaMCA1 may be involved in mediating the plant accumulation of ROS to influence plant resistance during the compatible interaction of wheat and *Pst*.

**TaMCA1 decreases the yeast resistance to H$_2$O$_2$.** Firstly, we examined the effects of TaMCA1 on the survival of yeast cells subjected to H$_2$O$_2$. As shown in Fig. 6a, the viability was severely reduced in the TaMCA1-transformed yeast cells grown on inducing medium with H$_2$O$_2$ compared with the cells grown on repressing medium with H$_2$O$_2$. Similar results were also obtained in the complementation experiment, the *yca1Δ* and *yca1Δ* expressing the empty vector (yeastΔ + EV) survived the H$_2$O$_2$ stimuli, and by contrast, both the wild type (W) and the *yca1Δ* expressing TaMCA1 (yeastΔ + TaMCA1) were shown to have a reduction of survival, respectively (Fig. 6b). Therefore, TaMCA1 could decrease yeast cell resistance to H$_2$O$_2$, and was able to partly complement the function of the YCA1.

No interaction between TaMCA1 and TaLSD1. *AtMCI* (At1g02170) interacts with the Arabidopsis lesion simulating disease 1 (*LSD1*, At4g20380) in yeast and transgenic Arabidopsis[^6^]. *TaLSD1* (EF553327) a negative regulator of programmed cell death, is involved in wheat resistance against stripe rust fungus[^2^]. To identify the interaction between TaMCA1 and TaLSD1, we used the yeast two-hybrid assay in this study. The transformants containing TaMCA1 and TaLSD1 plasmids were grown on selective double-dropout/-leucine-tryptophan (SD/-Leu-Trp) media. However, no clones were obtained on the selective quadruple dropout/-leucine-tryptophan-histidine-adenine (SD/-Leu-Trp-His-Ade) media containing 5-bromo-4-chloro-3-indoxyl-α-D-galactopyranoside (X-α-Gal) as a substrate. Our results revealed that there was no interaction between TaMCA1 and TaLSD1 (Fig. 7).

**Discussion**

In plant cell, cell death is often accomplished by biochemical and morphological hallmarks similar to those observed in animal apoptosis[^25^,^26^]. However, the orthologs of animal caspases, i.e., cysteiny1 aspartate-specific proteases, which are highly conserved in animal cells, have not yet been identified in plants[^27^]. In the last decade, a family of genes encoding cysteine-type C14 proteases that is more structurally similar to mammalian caspases than any other caspase-like proteases in plants were named metacaspases[^1^,^28^]. Within plant genomes, the *Arabidopsis thaliana* genome encodes three type I and six type II metacaspases, and the *Oryza sativa* sp. *japonica* genome encodes four type I and four type II metacaspases[^2^]. Recently, a number of reports have investigated the biological functions of plant type I metacaspases[^6^,^30^,^31^], however, little is known about the molecular mechanisms of metacaspases.

**Table 1.** Histological observations during the compatible interaction of wheat and CYR31 in knockdown wheat leaves. Treatment: The leaves were pre-inoculated with 1 × Fes buffer (MOCK), BSMV:γ, BSMV:TaMCA1-1 or BSMV:TaMCA1-2 followed by inoculation with *Puccinia striiformis* f. sp. *tritici* race CYR31. BSMV, barley stripe mosaic virus; hpi, hours post inoculation. All data (i.e., hyphal length, hyphal branches and colony size) are representative of the averages from at least 50 infection sites. The values within the same column followed by different letters were significantly different according to analysis of variance (ANOVA) (*P* < 0.05).

| Treatments          | Hyphal length (μm) | Hyphal branches | Colony size (× 1000 μm$^2$) | H$_2$O$_2$ area (× 1000 μm$^2$) |
|---------------------|--------------------|-----------------|-----------------------------|---------------------------------|
|                     | 24 hpi             | 48 hpi          | 24 hpi                      | 48 hpi                          | 120 hpi                        | 24 hpi                      | 48 hpi                          | 120 hpi                        |
| MOCK                | 36.01 ± 2.32a      | 62.55 ± 2.88a   | 2.22 ± 0.29a                | 2.39 ± 0.38a                    | 2.09 ± 0.22a                   | 0.59 ± 0.068                 | 1.80 ± 0.13a                   | 2.59 ± 0.28a                   |
| BSMV                | 36.21 ± 2.14a      | 63.12 ± 3.41a   | 2.23 ± 0.33a                | 2.41 ± 0.43a                    | 2.11 ± 0.25a                   | 0.58 ± 0.07b                 | 1.79 ± 0.14a                   | 2.63 ± 0.23a                   |
| BSMV:TaMCA1-1      | 33.83 ± 3.01a      | 54.78 ± 2.78b   | 2.13 ± 0.25a                | 2.45 ± 0.45a                    | 1.77 ± 0.89b                   | 0.91 ± 0.11a                 | 1.80 ± 0.11a                   | 2.72 ± 0.29a                   |
| BSMV:TaMCA1-2      | 34.69 ± 2.67a      | 53.15 ± 3.35b   | 2.22 ± 0.33a                | 2.39 ± 0.36a                    | 1.74 ± 0.18b                   | 0.83 ± 0.11a                 | 1.73 ± 0.11a                   | 2.69 ± 0.31a                   |
that regulate wheat plant resistance against Pst. In the present study, we obtained a novel *Triticum aestivum* metacaspase gene, *TaMCA1*, from *Triticum aestivum* cv. Suwon11. *TaMCA1* was predicted to be a member of the wheat type I metacaspase family, and contained a variable-sized N-terminal extension upstream of the p20 caspase-like domain and a short linker between the p20 and p10 domains.

Caspase (clan CD, family C14) is a member of the cysteine protease family and specifically cleaves after aspartate32. Caspases are synthesized inside the cells as inactive zymogens, but their activation can be specifically measured with synthetic substrates3. Our results showed that the synthetic substrate (Ac-YVAD-AMC) was not degraded by the recombinant *TaMCA1 in vitro*, although the substrate was degraded by the extract from wheat leaves (Fig. S3). A large amount of evidence demonstrate that recombinant metacaspases do not degrade synthetic substrates in vitro2,8,33, but caspase-like activity is found to be present in extracts from mosaic virus-infected tobacco leaves34 and barley (*Hordeum vulgare*) embryonic suspension cells35. Therefore, we speculate that *TaMCA1* may be activated through the proteolytic cleavage of zymogens or conformational changes inside plant cells induced by some signals.

Metacaspase has been identified as a major player in cell death.7,8,19,29,31,36,37, In the present study, our work showed that *TaMCA1* could suppress cell death induced by the Bax gene in *N. benthamiana* and wheat leaves (Figs 3 and 4). *TaMCA4* has also been shown to be involved in the cell death triggered by the Bax gene in *N. benthamiana* and wheat leaves39. Hence, we speculate that *TaMCA1* may be involved in wheat-*Pst* interaction as a negative cell death regulator. Furthermore, the expression level of *TaMCA1* was up-regulated (approximately 4-fold) at 72 hpi following challenge with *Pst* race CYR23 and remarkably up-regulated (approximately 38-fold) at 48 hpi following challenge with *Pst* race CYR31 (Fig. 1), which indicated that *TaMCA1* may be play an important role in the resistance mechanism against *Pst*.

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**Figure 6. Effects of expression of TaMCA1 in yeast cells.** (a) The yeast cells expressing *TaMCA1* or empty vector (EV) were spotted on solid medium. +EV, yeast cells expressing EV; −EV, yeast cells not expressing EV; +TaMCA1, yeast cells expressing *TaMCA1*; −TaMCA1, yeast cells not expressing *TaMCA1*. −H2O2, solid medium containing 0 mM H2O2; +H2O2, solid medium containing 1.5 mM H2O2. The final densities were 10⁶, 10⁵ and 10⁴ (cell/ml) following dilution with sterile water. (b) Survival of wild type (W), yeast metacaspase (YCA1) knock-out (yca1Δ), yca1Δ expressing empty vector (yca1Δ + EV) or yca1Δ expressing *TaMCA1* (yca1Δ + *TaMCA1*) in combination with or without 1.2 mM H2O2 treatment.
role in the compatible interaction of wheat and Pst. Therefore, we performed a knock down of TaMCA1 to determine its function during the wheat-Pst interaction. The average hyphal length at 48 hpi and the average colony size area at 120 hpi per infection site both decreased significantly in both TaMCA1-knockdown plants compared with the control plants (Table 1), and the Pst biomass in both TaMCA1-knockdown plants was significantly reduced at 120 hpi compared with the control plants (Table S1).

During plant-pathogen interactions, plants have developed a more sophisticated and efficient mechanism to counteract the spread of pathogen invasion, such as ROS bursts, protease activation. ROS accumulation in host cells is a plant defense response that is important for resistance against rust fungi in wheat38–40. In this study, the transcript levels of TaCAT, TaPOD and TaSOD were down-regulated in TaMCA1-knockdown plant after infection with Pst, especially at 24 hpi. Interestingly, the DAB staining of the TaMCA1-knockdown plants at the infection sites became more extensive at 24 hpi compared with those observed in the control plants (Table 1).

Similar observations have been reported in previous studies, AtMC1 mutant was hypersensitive to the salicylic acid agonist benzo(1,2,3)thiadiazole-7-carbothioic acid S-methylester, and accompanied by ROS accumulation41. A pepper (Capsicum annuum L.) metacaspase 9 (Camc9) was reported to be involved in the production of ROS during pathogen-induced cell death42. The yca1Δ survived in the presence of H2O2, and AtMC1, AtMC6 and AtMC8 were also able to complement the cell death functions of YCA143,44. In the present study, TaMCA1 in fission yeast decreased the resistance to H2O2 stimuli, and was also able to partly complement the function of the YCA1 (Fig. 6). These data strongly support the notion that TaMCA1 mediated the plant resistance to Pst by regulating ROS accumulation.

Previous reports have demonstrated that AtMC1 interacts with LSD1 in yeast and transgenic Arabidopsis45. However, there was no interaction between TaMCA1 and TaLSD1 based on the yeast two-hybrid assays (Fig. 7). These results suggested that the difference between TaMCA1 and AtMC1 is larger because of the wheat genome is much larger than those of species.

In summary, our study indicated that TaMCA1 would participate in the regulation of cell death only after the generation of sufficient signals, including mammalian Bax, Pst or H2O2. Furthermore, TaMCA1 was able to decrease plant resistance via the management of ROS accumulation during the compatible interaction of wheat and Pst.

**Methods**

**Plant materials and Pst isolate and treatments.** Two plants (Triticum aestivum cv. Suwon11 and N. benthamiana) and two Chinese Pst races (CYR23 and CYR31) were used in this study. Suwon11 exhibits a typical HR to CYR23 but is highly susceptible to CYR3142. The wheat seedlings were grown, inoculated and maintained following previously described procedures45. N. benthamiana was grown in the growth chamber at 22 °C under constant light and used for transient expression.

**RNA extraction and qRT-PCR.** For RNA isolation, the leaves were collected at 0, 12, 24, 48, 72 and 120 hpi with Pst. The time points were selected based on microscopic studies38,45. RNA was isolated from the wheat leaves using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. First-strand cDNA was synthesized using the GoScript Reverse-Transcription System (Promega Corp., Madison, WI, USA). The reverse transcription reactions were incubated with oligo(dT) primers at 42 °C for 1 h in total volumes of 20 μL. The primer design and qRT-PCR reactions were conducted as described previously45. To standardize the data, the wheat translation elongation factor TaEF-1a gene (Q03033) was used as an internal reference for the qRT-PCR analysis. Dissociation curves were generated for each reaction to ensure specific amplification. The threshold values (CtS) generated with the ABI PRISM 7500 Software Tool (Applied Biosystems) were employed.

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**Figure 7. Yeast two-hybrid assay to assess the interaction between TaMCA1 and TaLSD1.** AtMC1 interacted with LSD1 in a yeast two-hybrid assay, while there was no interaction between TaMCA1 and TaLSD1. (a) The diagram indicates the corresponding vector for the assay. (b) The transformants were selected through growth on selective double dropout/-leucine-tryptophan (SD/-Leu-Trp) media at 30 °C for 3 days. (c) Transformants were streaked on selective quadruple dropout/-leucine-tryptophan-histidine-adenine (SD/-Leu-Trp-His-Ade) media containing 5-bromo-4-chloro-3-indoxyl-α-D-galactopyranoside (X-α-Gal) as a substrate. The pair (pBD-TaEIL1, pAD) was provided as a positive control in the assay.
to quantify the relative gene expressions using the comparative 2^−ΔΔCT method. Three independent biological replicates were performed for each experiment.

**Sequence analysis, alignments and polymorphism analysis.** PROSITE Scan (http://prosite.expasy.org/scanprosite/) and Pfam (http://pfam.sanger.ac.uk/) were used to predict the conserved domains and motifs. CLUSTALW and DNAMAN6.0 were used to perform the multi-sequence alignments. The MEGA5.1 software was used to create the phylogenetic trees of the TaMCA1 members.

**Protein expression, purification and Activity assay.** Recombinant of TaMCA1 in pET-28a (+) was expressed in the *E. coli* strain BL21 (DE3) (Invitrogen). The cells were cultured in LB medium at 37 °C with ampicillin (50 μg/ml) to an OD600 of 0.4–0.6, and expression was subsequently induced by 0.5 mM isopropyl-β-d-thiogalactoside (IPTG) at 25 °C 200 rpm for 5 h. The protein was extracted and purified as described previously. Purified TaMCA1 was assayed with western blotting experiments. The fluorescent substrate Ac-TYVD-AMC (PharMingen; AMC7-amino-4-methylcoumarin, i.e., a substrate of caspase-1) was used to measure the activity of TaMCA1 as described previously.

**Subcellular localization of TaMCA1 in protoplasts.** In order to block out the chloroplast auto-fluorescence, the wheat seedlings for protoplast transformation were kept in the growth chamber at 16 °C without light for 7 days before using. Protoplast preparation and transformation were performed as described previously. The green channel shows the localization of TaMCA1-GFP; the red channel shows the localization of SLO2-DsRed, a mitochondrial marker protein (At2g13600). Bar = 20 μm.

**Overexpression of TaMCA1 in *N. benthamiana* and *T. aestivum*.** The reconstructed vectors PVX-eGFP, PVX-Avr1b, PVX-Bax and PVX-TaMCA1 were transformed individually into the *A. tumefaciens* strain GV3101 by electroporation. The transformants were grown on LB media plates with 30 μg/ml rifampicin, 30 μg/ml chloramphenicol and 30 μg/ml kanamycin. For the infiltration of the leaves, the *A. tumefaciens* strains carrying PVX-EV or PVX-Avr1b, PVX-eGFP, PVX-TaMCA1 or PVX-Bax were cultured in LB medium with rifampicin (30 μg/ml), chloramphenicol (30 μg/ml) and kanamycin (30 μg/ml) at 28 °C for 24–48 h. During the logarithmic phase, the cells were collected by centrifugation, washed twice with 10 mM MgCl2, and finally suspended to an OD600 of 0.8 with an infiltration media (10 mM MgCl2, 10 mM MES, pH 5.6, and 200 mM acetosyringone). Next, the cells were incubated at room temperature for 1–3 h before infiltration. *A. tumefaciens* strains carrying PVX-EV, PVX-eGFP, PVX-Avr1b or PVX-TaMCA1 were infiltrated into tobacco leaves using a syringe without a needle. The same infiltration site was challenged with a strain carrying PVX-Bax 24 h after the initial infiltration. The green fluorescence was detected in the PVX-eGFP treated leaves 72 h after infiltration and directly imaged on an Olympus BX-51 microscope (Olympus Corporation, Japan; excitation filter, 485 nm; dichromic mirror, 510 nm; barrier filter, 520 nm). Symptom development was monitored visually 3 to 8 days after infiltration.

For the particle bombardment assays, a great quantity of reconstructed plasmid (pUC-EV, pUC-Bax, pUC-Gus or pUC-TaMCA1) was prepared. Leaves from 4- to 6-week-old *N. benthamiana* plants were bombarded using the Bio-Rad He/1000 particle delivery system with a double-barreled extension attached, and leaves from 2- to 3-week-old *T. aestivum* plants were bombarded with single-barreled particle delivery as described previously. The DNA samples were prepared according to the shooting protocol described previously. After bombardment, the leaves were incubated at 28 °C for 2–3 days in darkness and then stained for 16–24 h at 28 °C using 5-bromo-4-chloro-3-indolyl-D-glucuronic acid (X-gal) at 0.8 mg/ml, 80 mM Na phosphate (pH 7.0), 8 mM Na2EDTA, 0.4 mM K3Fe(CN)6, 0.4 mM K4Fe(CN)6, 0.06% (vol/vol) Triton X-100 and 20% methanol. After bleaching the leaves using 100% methanol for many days until the blue spots could be observed clearly by microscopy, the initial infiltration. The green fluorescence was detected in the PVX-eGFP treated leaves 72 h after infiltration and directly imaged on an Olympus BX-51 microscope (Olympus Corporation, Japan; excitation filter, 485 nm; dichromic mirror, 510 nm; barrier filter, 520 nm). Symptom development was monitored visually 3 to 8 days after infiltration.

**Analysis of variance (ANOVA) was used to analyze the significant differences between the different treatments using SPSS software 18.0.**

**BSMV-mediated TaMCA1 gene silencing.** Capped in vitro transcripts were prepared from linearized plasmids that contained the tripartite BSMV genome using the mMessage mMACHINE T7 in vitro transcription kit (Ambion, Austin, TX, USA) following the manufacturer’s instructions. Suwon11 wheat seedlings at the two-leaf stage were prepared; the second leaf was treated with BSMV virus (BSMv-γ, BSMV:PDS, BSMV:TaMCA1-1 or BSMV:TaMCA1-2) as previously described and then maintained in a growth chamber at 23 ± 2 °C. The seedlings were mock inoculated with BSMV:PDS as a positive control, and 1 × FeS buffer was used a negative control. The fourth leaves then inoculated with fresh viruliferous insects of CYR31 at 10 dpi and subsequently sampled at 0, 24, 48 and 120 hpi for histological observation and RNA isolation. The infection types of stripe rust were examined at 15 dpi.

**Histological observations of the fungal growth.** A histopathological analysis was performed to characterize the cellular interaction between the wheat and *Pst*. BSMV-infected wheat leaves with *Pst* were sampled at 24, 48 and 120 hpi. Leaf segments cut from the inoculated leaves were fixed and decolorized with ethanol/trichloromethane (3:1 v/v) containing 0.15% (w/v) trichloroacetic acid for 3–5 days. The segments were soaked in saturated chloral hydrate until translucent and then stained with wheat germ agglutinin (WGA) conjugated to Alexa-488 (Invitrogen). The hyphal length, colony size and number of hyphal branches of stained tissues were examined under blue light excitation (excitation wavelength 450–480 nm and emission wavelength 515 nm) with an Olympus BX-53 microscope (Olympus Corporation, Japan) and calculated with the cellSens Entry software (Olympus Corporation, Japan) as described, *H2O2* was stained in situ using 3,3-diaminobenzidine (DAB; Amresco, Solon, OH, USA). The infection sites at which appressoria had
formed over the stomata were considered to have successful penetration, and at least 50 infection sites were examined on each of five randomly selected leaf segments per treatment.

**Fungal growth biomass during the wheat-Pst interaction.** To quantify the cDNA of Pst, the standard curves were first created with the Pst translation elongation factor gene via real-time PCR analysis. The threshold cycles (Cq) were plotted against the concentration of cDNA of the Pst race CYR31 (7.215, 3.608, 2.405, 1.804, 0.902 and 0.722 ng/μl) (Fig. S5). Dissociation curves were generated for each reaction to ensure specific amplification. The quantification of cDNA was performed using a RT-PCR System (Bio-Rad, Hercules, CA, USA). Three independent biological replicates were performed for each experiment.

**Expression of TaMCA1 in yeast.** The vector pREPS3X was used for the overexpression of Schizosaccharomyces pombe. In this system, thiamine was used as a repressor of the pREPS3X vector. For the assays of sensitivity to the H₂O₂ stimuli, the transformed cells were cultured in yeast medium with thiamine at 30 °C with an initial starting optical density at 600 nm (OD600) of 0.2. During the log phase, the cells were collected by centrifugation, washed thrice with sterile water and finally diluted to densities of 10⁶, 10⁵ and 10⁴ cell/ml using a blood-counting chamber and then assayed on yeast solid media plates with or without 1.5 mM H₂O₂ or thiamine. (B) The pYES2-TaMCA1 vector and empty vector were introduced into yca1Δ (KFY729) strain according to the user manual of pYES2 (Invitrogen). The transformed cells were assayed on yeast solid media plates with or without 1.2 mM H₂O₂.

**Yeast two-hybrid assay.** AtMCI (At1g02170), TaEIL1 Δ−yca1Δ and TaMCA1 were individually inserted into the pGBK7 vector (pBD); LSD1 (At4g20380) and TaLSD1 (EF553327) were individually inserted into the pGADT7 vector (pAD). The vectors (pBD-AtMCI, pAD-LSD1; pBD-TaEIL1, pAD; pBD-TaMCA1, pAD-TaLSD1) were co-transformed in pairs into the yeast strain AH109, and interactions were tested on selective double dropout/-leucine-tryptophan (SD/-Leu-Trp) media and subsequently on quadruple dropout/-leucine-tryptophan-histidine-adenine (SD/-Leu-Trp-His-Ade) media containing 5-bromo-4-chloro-3-indoxyl-α-D-galactopyranoside (X-Gal/Gal) as a substrate according to the manufacturer's instructions.

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Author Contributions

Y.H., X.W. and Z.K. designed the research. Y.H., K.W., H.L., X.D. and C.T. performed the experiments and analyzed data. Y.H. and X.W. wrote the manuscript.

Additional Information

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