TaBln1, a member of the Blufenfensin family, negatively regulates wheat resistance to stripe rust by reducing Ca\(^{2+}\) influx

Shuangyuan Guo,2,3 Yanqin Zhang,1,3 Min Li,2,3 Peng Zeng,1,3 Qiong Zhang,3 Xing Li,1,3 Quanle Xu,1 Tao Li,4 Xiaojie Wang,2,3 Zhensheng Kang,2,3 and Xinmei Zhang1,3,*

1 College of Life Sciences, Northwest A&F University, Yangling, Shaanxi 712100, China
2 College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100, China
3 State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University, Yangling, Shaanxi 712100, China
4 Jiangsu Key Laboratory of Crop Genomics and Molecular Breeding, Agricultural College of Yangzhou University, Yangzhou, Jiangsu 225009, China

*Author for correspondence: xinmeizhang@nwsuaf.edu.cn
†Senior author
S.Y.G., Z.S.K., X.J.W., and X.M.Z. conceived and designed the experiments; Q.Z., Q.L.X., and T.L. contributed to the original concept of the project. S.Y.G., Y.Q.Z., M.L., P.Z., and X.L. performed the experiments; S.Y.G. and X.L. performed the data analyses. S.Y.G. wrote the manuscript. Z.S.K., X.J.W., and X.M.Z. revised the manuscript.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/general-instructions) is Xinmei Zhang (xinmeizhang@nwsuaf.edu.cn).

Abstract
Blufenfensin (Bln1) has been identified as a susceptibility factor of basal defense mechanisms which is unique to the cereal grain crops barley (Hordeum vulgare), wheat (Triticum aestivum), rice (Oryza sativa), and rye (Secale cereale). However, the molecular mechanisms through which Bln1 regulates the wheat immune response are poorly understood. In this study, we found that TaBln1 was significantly induced by Puccinia striiformis f. sp. tritici (Pst) virulent race CYR31 infection. Knockdown of TaBln1 expression by virus-induced gene silencing reduced Pst growth and development, and enhanced the host defense response. In addition, TaBln1 was found to physically interact with a calmodulin, TaCaM3, on the plasma membrane. Silencing TaCaM3 with virus-induced gene silencing increased fungal infection areas and sporulation and reduced wheat resistance to the Pst avirulent race CYR23 (incompatible interaction) and virulent race CYR31 (compatible interaction). Moreover, we found that the accumulation of TaCaM3 transcripts could be induced by treatment with chitin but not flg22. Silencing TaCaM3 decreased the calcium (Ca\(^{2+}\)) influx induced by chitin, but silencing TaBln1 increased the Ca\(^{2+}\) influx in vivo using a noninvasive micro-test technique. Taken together, we identified the wheat susceptibility factor TaBln1, which interacts with TaCaM3 to impair Ca\(^{2+}\) influx and inhibit plant defenses.

Introduction
Plant genes that facilitate pathogen infection and support compatibility can be considered susceptibility (S) genes (Lapin and Ackerveken, 2013; Schie and Takken, 2014). Mutation or loss of S genes could result in a more broad-spectrum and durable natural resistance in plants (Pavan et al., 2010; Schie and Takken, 2014). Some successful examples are the use of mildew resistance locus O (MLO) mutations in barley (Hordeum vulgare), wheat (Triticum aestivum), and tomato (Solanum lycopersicum), which confer heritable resistance to
powdery mildew by preventing TaBln1

Generation of pivotal signaling molecules. CaMs and CaM-like (CML) proteins are involved in this signaling functioning as Ca^{2+} sensors and mediating the synthesis of NO (Ma and Berkowitz, 2011). CaMs and CMLs constitute a large family of Ca^{2+} binding and signaling proteins in plants. Among these proteins, CaM, containing a pair of Ca^{2+}-binding EF-hand motifs, is the most thoroughly characterized Ca^{2+} sensor in plants (Bouché et al., 2005; Defalco et al., 2009). Plant genomes contain multiple loci that encode conserved CaM isoforms. For example, seven distinct genes encode four protein isoforms (CaM2/CaM3/CaM5, CaM1/CaM4, CaM6, and CaM7) in Arabidopsis (Arabidopsis thaliana) (McCormack and Braam, 2003). It has been reported that CaMs are involved in innate immunity in plants, which can transmit the initial signal of cytosolic Ca^{2+} elevation to downstream targets in a signal transduction cascade (Ma and Berkowitz, 2007). Pathogen infection results in induction and/or suppression of plant CaM isoforms (Garcia-Brugger et al., 2006). Manipulating plant CaM expression affects basal resistance to a range of pathogens (Heo et al., 1999; Takabatake et al., 2007). For example, the silencing of specific pathogen-induced CaM isoforms in tomato results in enhanced susceptibility to virulent necrotrophic bacteria and fungi (Takabatake et al., 2007). The expression of ScaM4 and ScaM5 in transgenic tobacco and Arabidopsis lead to increased pathogenesis-related gene expression and enhanced resistance to bacterial, fungal, and viral pathogens (Zhu et al., 2010). CaM may be a key player in transducing pathogen-induced Ca^{2+} increase to the downstream components of defense signaling and contributing to plant defense responses.

Wheat (T. aestivum L.) is one of the most important dietary crops in the world. While growing, wheat undergoes continuous exposure to abiotic and biotic stresses in the natural environment. Puccinia strififormis f. sp. tritici (Pst), an obligate biotrophic pathogen, is the causal agent of wheat stripe rust and has become the largest biotic limitation to wheat production and the global food supply (Liu et al., 2016; Schuessinger, 2017). Genetic control is the most cost-effective strategy for reducing the threat of this disease (Ellis et al., 2014). Recent studies have indicated that broad-spectrum resistance can result from loss of function or a mutation of a susceptibility factor (Qiang et al., 2021). Therefore, a better understanding of the mechanisms at the molecular level is of great importance to improve disease resistance in wheat.

We identified and functionally characterized one Blufensin gene TaBln1 on chromosome 4A, and assessed its role in wheat resistance to Pst. In addition, we demonstrated that TaBln1 interacts with one CaM, TaCaM3, which was shown to play a positive role in resistance to stripe rust in wheat. Concurrently, a noninvasive micro-test technique and chitin-treatment experiments showed that transient silencing of TaCaM3 led to a decrease in Ca^{2+} influx in wheat mesophyll cells; however, silencing TaBln1 led to a substantial increase in Ca^{2+} influx. Our results demonstrate that
TaBln1 negatively regulates wheat resistance to Pst by affecting the balance between TaCam3 and Ca2+, and TaCam3 might be the target for TaBln1 in the wheat response to Pst infection. Our results provide insights that may lead to improved understanding of the roles of susceptibility factors in plant resistance.

Results

TaBln1 is significantly induced by Pst infection
TaBln1 was isolated from the transcriptome of wheat cultivar Suwon 11 infected by virulent Pst CYR31. BLAST analyses of Ensemble Plants database revealed that three copies of TaBln1 were located on chromosomes 4A, 5B, and 5D, but no complete sequence information was found. Subsequently, the copies of TaBln1 were obtained by genomic PCR (Polymerase Chain Reaction) with specific primers (Supplemental Table S1) according to the Ensemble Plants database. The three copies shared 97.33% DNA identity and 97.56% protein identity (Supplemental Figure S1), indicating that they may possess identical biological functions. Sequence alignment showed that TaBln1-4A share the same protein sequence as TaBln1 identified in previous research (Meng et al., 2009).

To investigate whether TaBln1 participates in the wheat response to Pst, we used reverse transcription-quantitative PCR (RT-qPCR) to analyze the relative transcript profiles of TaBln1 in the Pst avirulent race CYR23 and the virulent race CYR31 inoculated wheat at different time points. The transcript level of TaBln1 was increased at 12-h and 24-h postinoculation (hpi) in wheat leaves challenged with the Pst avirulent race CYR23 (incompatible interaction). The expression of TaBln1 was upregulated at 12, 24, 48, and 72 hpi in wheat leaves challenged with the Pst virulent race CYR31 (compatible interaction) and the highest TaBln1 transcript level was approximately 24-fold at 24 hpi (Figure 1). These data suggested that TaBln1 may participate in the interaction between wheat and Pst at the early stage of the Pst incubation period.

Silencing of TaBln1 reduces wheat susceptibility to Pst
To determine the role of TaBln1 in the wheat–Pst interaction, we used a BSM virus-induced gene silencing (VIGS) system to knockdown the expression of TaBln1. Photobleaching was observed in plants inoculated with BSMV:TaPDS (wheat phytoene desaturase gene), and mild chlorotic mosaic symptoms were displayed in other BSMV-infected plants with no evident defects (Figure 2A). BSMV:TaPDS was used as a positive control to indicate the efficacy of the VIGS system. Thereafter, the Pst CYR31 was used to inoculate the BSMV-treated plants. The disease phenotype showed that there were fewer uredinia on TaBln1-knockdown plants at 14-day postinfection (dpi) than on the control plants (Figure 2B). Moreover, the fungal biomass was significantly lower on TaBln1-knockdown plants at 5, 7, and 10 dpi than on the control plants, which is consistent with the disease phenotype (Figure 2C). Standard curves (Supplemental Figure S2) for biomass were generated using qPCR with total genomic DNA (Yin et al., 2009). The transcriptional levels of TaBln1 in BSMV:TaBln1-inoculated leaves were ~40%–95% lower at 0, 24, 48, and 120 hpi than in control plants (Figure 2D), indicating that the expression of TaBln1 was substantially knocked down by VIGS.

Knockdown of TaBln1 expression reduces Pst growth and development and enhances the host defense response
To quantify the reduced disease phenotype in TaBln1-knockdown plants, we also microscopically examined fungal growth and development in TaBln1-silenced leaves (Figure 3A). The number of hyphal branches (HBS) and haustoria at 24 hpi and the length of hyphae at 48 hpi were significantly reduced in TaBln1-silenced plants relative to control plants inoculated with BSMV:γ (Figure 3B and C). Compared to the control plants, the infection areas of Pst at 120 hpi also decreased by silencing of TaBln1 (Figure 3D). Moreover, to further analyze the host response, we measured the accumulation of H2O2 in TaBln1-knockdown plants with 3,3-diaminobenzidine (DAB) staining. Our results revealed that the area that contained H2O2 was significantly larger in TaBln1-silenced leaves than in the control leaves at 24, 48, and 120 hpi (Supplemental Figure S3). These histological results indicate that the silencing of TaBln1 restricted the growth and development of Pst and enhanced the resistance of wheat to Pst.
TaBln1 interacts with TaCaM3

BLN1 and BLN2 were found to interact with a CaM in barley (Xu et al., 2015), suggesting that TaBln1 may interact with a CaM in wheat. To confirm this prediction, TaCaM3, a homologue of barley CaM, was cloned in wheat cultivar Suwon11. BiFC was first used to detect whether TaBln1 interacts with TaCaM3 in transiently transformed Nicotiana benthamiana leaves. Strong fluorescence signals were obtained in the interaction between TaBln1 and TaCaM3 after 48 hpi (Figure 4A). However, there were no fluorescence signals for the co-expression of NE-TaBln1 and the empty vector CE in N. benthamiana leaves (Figure 4A). To further investigate their physical association with co-immunoprecipitation (Co-IP) assays, we transiently co-expressed TaBln1 fused with the GFP tag and TaCaM3 fused with the HA tag in N. benthamiana. As expected, TaCaM3 was co-immunoprecipitated with TaBln1 but not with GFP (Figure 4B). Next, their interaction was further confirmed in split-luciferase (LUC) assays. The nLUC fusion of TaBln1 and cLUC fusion of TaCaM3 were co-expressed in N. benthamiana. Only the area co-expressing TaBln1-nLUC and TaCaM3-cLUC had a strong luminescence signal; no luminescence signals appeared in the area co-expressing TaBLN1-nLUC and GUS-cLUC (Figure 4C). In addition, the in vitro interaction between TaBln1 and TaCaM3 was tested using a pulldown assay with TaBln1 fused with GST tag and TaCaM3 fused with His tag. When detected with anti-His monoclonal antibodies, TaCaM3 was detected in Western blots of proteins eluted from TaBln1-GST beads, but no TaCaM3 was detected from free GST beads (Figure 4D). These data confirmed that TaBln1 interacts with TaCaM3.

The conserved cysteines in CaM targets are important for CaM target interactions (Moore et al., 1999; Xu et al., 2015). To study the effect of conserved cysteines on the interaction between TaBln1 and TaCaM3, the two conserved cysteines of TaBln1 were mutated into glycine (Supplemental Figure S4, A...
and B). Then Co-IP and split-LUC assays were used to verify their interaction again. The result of Co-IP showed that the interaction between TaBln1 and TaCaM3 was eliminated (Figure 4B), and split-LUC assays showed that there was only a slight interaction between TaBln1 and TaCaM3, the signal intensity of the interaction was substantially reduced (Figure 4C). These data confirmed that the conserved cysteines in TaBln1 are important for the TaBln1–TaCaM3 interaction. This is also consistent with the previous results (Xu et al., 2015).

TaCaM3 co-localizes with TaBln1 to the plasma membrane

To determine the subcellular localization of TaBln1, we first analyzed the protein sequence for TaBln1. TaBln1 contained a signal peptide (1–29 aa) and a putative transmembrane domain (Supplemental Figure S5), indicating that it might be localized to the plasma membrane. To confirm this prediction, TaBln1-GFP and a plasma membrane marker protein, TaWpi6-mCherry, were co-expressed in N. benthamiana leaves. The GFP fluorescent signals of TaBln1-GFP were detected in the plasma membrane and nuclear membrane; meanwhile, the green fluorescence substantially overlapped with the red fluorescence of TaWpi6-mCherry (Figure 5A). To further validate the localization of TaBln1, plasmolysis was induced by the addition of 800-mM mannitol, and then, clear plasma membrane and nuclear membrane signals were observed for TaBln1-GFP (Figure 5B), indicating that TaBln1 was localized to the plasma

Figure 3 Knocking down TaBln1 reduces growth and development of Pst race CYR31. A, Fungal structures in wheat leaves infected with BSMV and Pst were visualized with WGA and the fungal structures were observed under a fluorescence microscope. SV, substomatal vesicle; IH, infection hypha; H, haustoria. Bar = 20 μm. B, The average number of HBs and haustoria (H) of Pst in each infection site were counted at 24 hpi. C, The hyphal length of Pst in TaBln1-silenced plants at 48 hpi. Hyphal length is the average distance to the tip of the hypha from the intersection of the sub-stomatal vesicle and the hypha was measured using CellSens Entry software (unit in μm). D, The infection area at 120 hpi was calculated using DP-BSW software (units of μm²). B–D, Means ± SD were calculated from 50 infection sites and were represented as solid lines in the picture. Three independent biological experiments were performed. Asterisks indicate a significant difference ( *P ≤ 0.05, **P ≤ 0.01) from BSMV γ inoculated plants using the Student’s t test.
membrane and nuclear membrane. In addition, in view of the interaction between TaBln1 and TaCaM3, we used TaCaM3-GFP and TaBln1-mCherry to determine whether TaBln1 co-localized with TaCaM3 to the plasma membrane and nuclear membrane. We determined the subcellular localization of TaCaM3, which similar to the free GFP, was detected throughout the cytosol, plasma membrane, and nucleus (Supplemental Figure S6). However, the results of co-localization showed that the red fluorescence of TaBln1-mCherry substantially overlapped with the green fluorescence of TaCaM3-GFP in the plasma membrane but the free mCherry overlapped with the TaCaM3-GFP in the nucleus, cytosol and plasma membrane (Figure 5C). This suggests that TaBln1 mainly co-localized with TaCaM3 to the plasma membrane.

Silencing of TaCaM3 reduces wheat resistance to Pst
To obtain direct evidence for the function of TaCaM3, we first assayed the expression of TaCaM3 at different levels of Pst infection. The six copies of TaCaM3 on chromosomes 2A, 2B, 2D, 4A, 4B, and 4D share 95.5% similarity in nucleotide sequence (Supplemental Figure S7) and with the majority sharing identical protein sequence (Supplemental Figure S8), which led to the design of conservative TaCaM3 primers for RT-qPCR. The transcription of TaCaM3 was significantly induced in both compatible and incompatible interactions at 12 and 24 hpi. The highest TaCaM3 transcript level was approximately three-fold in the incompatible interaction at 48 hpi (Supplemental Figure S9), suggesting that TaCaM3 plays a role in the interaction between wheat and Pst.

To determine the function of TaCaM3 in the wheat–Pst interaction, we silenced TaCaM3 using VIGS. When TaCaM3-knockdown plants were inoculated with CYR23, conspicuous HR was elicited on leaves that had previously been infected with BSMV:γ and BSMV:TaCaM3 (Figure 6A). However, a slight sporulation of Pst emerged around the necrotic regions on leaves infected with BSMV:TaCaM3 at 14 dpi (Figure 6A). Although there was no obvious difference between the BSMV:TaCaM3 and BSMV:γ plants in fungal biomass at 10 dpi, the fungal biomass was significantly increased in BSMV:TaCaM3 leaves at 5 and 7 dpi, relative to control plants (Figure 6B). Relative to leaves inoculated with BSMV:γ, the transcription of the endogenous TaCaM3 was successfully silenced in the fourth leaves of BSMV:TaCaM3 plants (Figure 6C). Moreover, we microscopically examined TaCaM3-silenced leaves infected with Pst CYR23. The length of the hyphae and the numbers of HBs, haustorial mother cells (HMCs), and haustoria at 24 hpi were significantly greater in the TaCaM3-silenced plants than in the control plants (Figure 6, D and E). At 120 hpi, the infection areas of Pst in TaCaM3-silenced plants were much larger than those in the control plants.
plants (Figure 6F). In addition, to study the effects of the knockdown of TaCaM3 on the expression of defense-related genes in wheat seedling leaves inoculated with CYR23, we assayed the expression of three PR genes in TaCaM3-silenced plants. The transcript levels of pathogenesis-related genes TaPR1, TaPR2, and TaPR5 were reduced at 24 and 48 hpi in the TaCaM3-knockdown plants (Figure 6G), indicating that silencing of TaCaM3 may reduce the resistance of wheat.

Because of the high transcriptional levels of TaCaM3 in compatible interactions, we also used the CYR31 to inoculate TaCaM3-knockdown plants. Although all leaves inoculated with CYR31 produced numerous uredospores (Figure 7A), the fungal biomass was significantly increased in BSMV:TaCaM3 leaves at 5 dpi compared to the control plants (Figure 7B). The silencing efficiency analyses performed by RT-qPCR indicated that TaCaM3 was significantly knocked down in leaves inoculated with CYR31 at 0, 24, 48, and 120 hpi (Supplemental Figure S10). Moreover, histological observations showed that the hyphal length at 24 hpi (Figure 7C) and infection areas of Pst at 120 hpi (Figure 7D) were also greater after the silencing of TaCaM3 than plants inoculated with BSMV:γ. These results also confirmed that silencing of TaCaM3 reduced the wheat resistance to Pst, and TaCaM3 may contribute to the basal immune response to Pst in wheat.

**TaBln1 impairs Ca2+ influx possibly by interacting with TaCaM3**

CaMs, as a universal Ca2+ sensor, can transmit the initial signal of cytosolic Ca2+ elevation (upon pathogen perception).
to downstream targets in a signal transduction cascade (Garcia-Brugger et al., 2006). In this study, to further study the function of TaCaM3, two PAMPs (Pathogen Associated Molecular Patterns, chitin and flg22) treatments were selected to measure the TaCaM3 transcript levels by RT-qPCR. The results of RT-qPCR showed that TaCaM3 was only upregulated upon chitin treatment (Figure 8A) but did not change upon flg22 treatment (Figure 8B). This indicated that chitin treatment could be used for further study.

We also investigated whether TaCaM3 may affect Ca^{2+} influx by measuring the dynamics of Ca^{2+} flux in mesophyll cells after treatment with chitin in vivo, using a noninvasive micro-test technique. All of the leaves tested were clearly responsive to chitin treatment. In response to chitin stimulation, the mesophyll cells of control plants inoculated with BSMV:γ exhibited robust Ca^{2+} influx; however, little change was seen in TaCaM3 knockdown plants (Figure 9A). Because TaBln1 can interact with TaCaM3, we speculated that TaBln1 might also affect the Ca^{2+} influx by influencing TaCaM3. To test this hypothesis, we also measured the Ca^{2+} influx in TaBln1 knockdown plants. The results showed that the mesophyll cells of plants inoculated with

Figure 6  Silencing TaCaM3 reduces wheat resistance to Pst CYR23. A, Disease phenotypes of the fourth leaves pre-inoculated with BSMV constructs and then challenged with Pst CYR23. B, The biomass ratio (Pst/wheat) measurements of total DNA extracted from BSMV-treated wheat leaves infected by CYR23 at 5, 7, and 10 dpi. C, The relative expression levels of TaCaM3 in leaves inoculated with CYR23 were assayed by RT-qPCR at 0, 24, 48, and 120 hpi. D, The hyphal length of Pst in TaCaM3-silenced plants at 24 hpi. E, The number of HB, HMC, and H of Pst in each infection site were counted at 24 hpi. Data from 90 infection sites of three biological replicates were pooled, horizontal box width represents the proportion of number at the ordinate value. F, TaCaM3-silenced plants show a significant increase in infection unit area at 120 hpi. G, Relative expression of the marked defense-related genes in the fourth leaves at 24 and 48 hpi. Transcript levels were quantified by RT-qPCR and normalized with TaEF-1α. B, C, and G, Values represent the means ± SD of three independent samples. D and F, Means ± SD were calculated from 90 infection sites of three biological replicates and were represented as solid lines in the picture. Asterisks indicate a significant difference (*P < 0.05, **P < 0.01) from BSMV:γ inoculated plants using the Student's t test.
BSMV:TaBln1, but not those in control plants, exhibited stronger and more rapid Ca\(^{2+}\) influx (Figure 9B). These results suggest that TaCaM3 could affect Ca\(^{2+}\) influx, and this ability was impaired by interaction with TaBln1.

**Discussion**

The use of loss-of-function mutations in S genes can save expenditure on pesticides and fungicides and eliminate their negative impacts on the environment. Hence, the study of S genes is of great interest for engineering plant disease resistance. In this study, we identified TaBln1 in wheat, which was significantly expressed in the early stages of infection, displaying higher transcript levels in compatible interactions than incompatible interactions. Thus, we speculated that TaBln1 may play a role in wheat susceptibility. Similar findings regarding Bln genes have been reported in barley: the overexpression Bln1 and Bln2 by BSMV significantly increased susceptibility of barley to Bgh (Meng et al., 2009; Xu et al., 2015), whereas the silencing of Bln1 enhanced barley resistance in compatible interactions (Meng et al., 2009).

In this study, we found that silencing TaBln1 limited the hyphal growth and fungal colony areas of Pst and increased ROS accumulation of wheat, resulting in a reduction in the number of Pst uredinia. Thus, we demonstrated that the function of the TaBln1 gene is similar to the Bln1 and Bln2 genes of barley, which played a negative regulatory role in the interaction between wheat and Pst. Interestingly, the Bln genes may be unique to the cereal grain crops barley, wheat, rice, and rye (Meng et al., 2009). The identification of TaBln1, as a susceptibility factor, would provide a preliminary target in the future for editing Bln genes with CRISPR to achieve durable disease control.

Previous studies have indicated that BLN family members in barley are cysteine-rich small peptides (Meng et al., 2009),

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**Figure 7** Knocking down TaCaM3 enhances wheat susceptibility to Pst CYR31. A, Disease phenotypes of the fourth leaves pre-inoculated with BSMV constructs and then challenged with Pst CYR31. B, The biomass ratio (Pst/wheat) measurements of total DNA extracted from BSMV-treated wheat leaves infected by CYR31 at 5 dpi based on RT-qPCR. Values represent the means ± SD of three independent samples. C, Significant increase in the hyphal length of Pst in TaCaM3-silenced plants at 24 hpi. D, TaCaM3-silenced plants show a significant increase in infection unit area at 120 hpi. C and D, Means ± SD were calculated from 90 infection sites of three biological replicates and were represented as solid lines in the picture. Asterisks indicate a significant difference (**P < 0.01) from BSMV:γ inoculated plants using the Student’s t test.
and both BLN1 and BLN2 interact with CaM (Xu et al., 2015). In this study, we compared the amino acids (AA) of Bln1 in wheat with Bln1 and Bln2 in barley and found that they all had conserved cysteines (Supplemental Figure S11). These conserved cysteines in CaM targets are important for CaM target interactions (Moore et al., 1999). Thus, we speculated that TaBln1 possessed a function somewhat similar to HvBln1 and HvBln2, which may interact physically with CaM. Interestingly, our results of BiFC, pull-down, LUC, and Co-IP analyses support this speculation. This interaction between TaBln1 and TaCaM3 implies that they might be regulated differently during wheat–Pst interactions. We speculate that TaBln1 negatively regulates immunity in wheat by affecting the function of TaCaM3.

CaMs play a critical role in plant defense (Takabatake et al., 2007; Zhu et al., 2010). TaCaM3, a CaM, is a highly conserved intracellular Ca\(^{2+}\) sensor (Bouché et al., 2005; Defalco et al., 2009). In this study, we identified TaCaM3 as a positive regulator of basal immunity against the Pst fungus, and we found that transiently silencing TaCaM3 in wheat decreased resistance to fungal infection, which increased areas of infection and sporulation. Numerous studies have suggested a key role for CaM-mediated Ca\(^{2+}\) signaling in plant growth and stress responses (Liu et al., 2003; Du et al., 2009). Thus, we speculated that TaCaM3 plays a positive regulatory role in the interaction between wheat and Pst through TaCaM3-mediated Ca\(^{2+}\) signaling. To test this hypothesis, chitin was selected to elicit early immune responses in the plant.

Intracellular Ca\(^{2+}\) transients during plant–pathogen interactions are necessary early events that lead to local and systemic acquired resistance (Lecourieux et al., 2006). Ca\(^{2+}\) binding to CaMs regulates cellular processes directly by binding to specific DNA sequences and modulates gene expression indirectly by interacting with other proteins in a Ca\(^{2+}\)-dependent manner and modulating their activity (Reddy et al., 2011). Therefore, intracellular Ca\(^{2+}\) transients are essential for CaMs to function in plant defense signaling pathways. In this study, we measured the dynamics of Ca\(^{2+}\) influx in mesophyll cells after treatment with chitin in vivo using the noninvasive micro-test technique. The results indicated that transiently silencing TaCaM3 decreased the Ca\(^{2+}\) influx induced by chitin, consistent with the results of VIGS experiments. We speculated that transient silencing of TaCaM3 affected the balance between Ca\(^{2+}\) binding and Ca\(^{2+}\) influx and ultimately weakened the disease resistance of wheat plants. Previous studies have indicated that CaM induces a net Ca\(^{2+}\) influx into protoplasts, leading to an increase in cytoplasmic Ca\(^{2+}\) levels (Wang et al., 2009a). In line with this finding, our results indicated that TaCaM3 affected Ca\(^{2+}\) influx. In addition, we proved that TaBln1 could interact with TaCaM3, so we speculated that TaBln1 might also affect Ca\(^{2+}\) influx by affecting the function of TaCaM3. Interestingly, transiently silencing TaBln1 produced a stronger and more rapid Ca\(^{2+}\) influx, which was also consistent with the results of VIGS. Recently, similar findings on the relationship between Ca\(^{2+}\) influx and immune responses have been reported. For example, by comparing the Ca\(^{2+}\) influx in the mesophyll cells of WT and transgenic rice plants after chitin or flg22 treatments, a study found that activated OsCNGC9, a cyclic nucleotide-gated channel protein, induced extracellular Ca\(^{2+}\) influx and then triggered a ROS burst and PTI-related gene expression, which ultimately led to enhanced disease resistance in rice (Wang et al., 2019). Therefore, we speculated that TaCaM3 could affect Ca\(^{2+}\) influx, and this ability was impaired by the interaction with TaBln1. TaCaM3 could not effectively transmit the initial signal of cytosolic Ca\(^{2+}\) elevation to downstream targets in a signal transduction cascade, which led to a weakening of plant disease resistance. To confirm our speculation, the relative expression of TaCaM3 in TaBln1-silenced leaves was determined by RT-qPCR. As expected, silencing of TaBln1
increased the accumulation of TaCaM3 transcripts, but silencing of TaCaM3 had no influence on the expression of TaBln1 (Supplemental Figure S12). These data confirmed that TaCaM3 was indeed affected by TaBln1, and which seems to affect the transmission of Ca2+ signals.

In addition, analyses of the TaBln1 sequence found that TaBln1 is a three-copy gene located on chromosomes 4A, 5B, and 5D. Because the reciprocal translocation of 4A with 5AL, also referred to as T(4AL;5AL)1, exists in diploid T. monococcum (Dubcovsky et al., 1996), the evolution of the wild emmer wheat chromosome 4A must have started with this translocation. In the evolution of wheat chromosomes 4A, 5A, and 7A, wheat chromosome 4A was subjected to the reciprocal translocation T(4AL;5AL)1 (Dvorak et al., 2018). Analyses of the TaBln1 protein structure found that although TaBln1 contains a signal peptide (1–19 aa), it also has a putative transmembrane domain (Supplemental Figure S4). In Arabidopsis, receptor kinase FLS2 contains a signal peptide and a transmembrane domain, which can be targeted to the plasma membrane (Gómez-Gómez and Boller, 2000). OsCERK1 (chitin elicitor receptor kinase), which encodes a receptor-like kinase containing a signal peptide, an extracellular domain, a transmembrane region, and an intracellular Ser/Thr kinase domain, is also localized to the plasma membrane (Shimizu et al., 2010). Therefore, we speculate that TaBln1 possess a similar localization to the aforementioned proteins. As expected, this study confirmed the localization of TaBln1 on the plasma membrane and nuclear membrane through N. benthamiana subcellular localization. At the same time, a plasmolysis experiment confirmed the localization of TaBln1 (Figure 5B; Supplemental Figure S13). This result is different from the localization of BLN1 and BLN2, which can be secreted to the apoplast in barley (Xu et al., 2015). Consistent with the negative control free GFP but in contrast with the positive control SP (TaPR1a)—GFP (Bi et al., 2020), TaBln1, after
plasmolis, was still localized to the membrane, and no apoplastic signals were observed (Supplemental Figure S13).

In order to exclude the influence caused by the heterologous systems, HvBln1 was expressed in N. benthamiana. We found that HvBln1 could be secreted into the apoplast (Supplemental Figure S14, A and B), while TaBln1 was not (Supplemental Figure S13). To explore the cause of these results, the aa sequences of HvBln1 and TaBln1 were first compared. The result showed 4 aa differences located at 5 (signal peptide region), 30, 35, and 47, respectively (Supplemental Figure S14C). Subsequently, we mutated the 4 aa of TaBln1 into aa consistent with HvBln1, respectively, and then performed localization analysis in N. benthamiana. Interestingly, the localization of TaBln1 was consistent with that of HvBln1 after mutation of the fifth amino acid, and fluorescence could be observed in the apoplast after plasmolysis. However, other mutations of TaBln1 had no substantially changes in its localization (Supplemental Figure S14D). We speculate that the secretion of a protein is closely related to the aa sequence of the signal peptide, which can provide ideas for the strategy of modifying protein localization in the future. In addition, we speculated that the localization of TaBln1 may be related to its negative regulatory function in the interaction between wheat and Pst. The results of co-localization showed that TaCaM3 and TaBln1 were co-localized to the plasma membrane, and the BIFC results showed that the fluorescence signal is mainly distributed at the plasma membrane. Therefore, we speculate that the interaction between TaBln1 and TaCaM3 mainly occurs at the plasma membrane. TaBln1 affects the Ca\(^{2+}\) influx modulated by TaCaM3, possibly by hijacking TaCaM3 to the plasma membrane.

**Conclusions**

This study reveals the potential molecular mechanism of TaBln1 in the wheat–Pst interaction. In uninoculated wheat leaves (Figure 10A), the expression of TaBln1 is maintained at a low level. TaCaM3 functions normally in binding Ca\(^{2+}\) and it may be transferred to downstream targets to activate the immune responses. During Pst infection of wheat (Figure 10B), the expression of TaBln1 may be upregulated and localized to the plasma membrane. The interaction between TaBln1 and TaCaM3 results in the accumulation of TaCaM3 on the plasma membrane. This, in turn, affects the balance between Ca\(^{2+}\)-binding and Ca\(^{2+}\) influx, thereby reducing the transmission of Ca\(^{2+}\) signals to downstream targets, and ultimately weakening the disease resistance of wheat. Obviously, a better understanding of the physiological importance of the cytosolic Ca\(^{2+}\) changes in plant–pathogen interactions will involve the identification and functional analysis of downstream targets of cytosolic Ca\(^{2+}\). Thus, most intracellular target proteins that sense and relay Ca\(^{2+}\) signatures toward the appropriate defense responses remain possible objects of future testing.

**Materials and methods**

**Plant materials, inoculation, and treatment**

Wheat (T. aestivum cv. Suwon 11), N. benthamiana, and two Pst pathotypes, CYR23 and CYR31, were used in this study. Suwon 11, carry the YrSu resistance gene, exhibits a high resistance to CYR23 but high susceptibility to CYR31 (Cao et al., 2003). Wheat seedling cultivation and inoculation with Pst were performed as described previously (Kang et al., 2002). Leaves were collected at 0, 12, 24, 48, 72, and 120 hpi for RNA isolation (Wang et al., 2007). The N. benthamiana was grown at 22°C and used for transient expression.

**Genomic DNA, total RNA extraction, and cDNA synthesis**

Genomic DNA was extracted using the cetyltrimethylammonium bromide method (Porebski et al., 1997). The total RNA from the wheat leaves was isolated using a Quick RNA isolation Kit (TIANGEN BIOTECH, Beijing, China), according to the manufacturer’s instructions. The extracted RNA was reversely transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) and oligo (dT)\(_{18}\) primer following the manufacturer’s protocol.

**Cloning of TaBln1 and TaCam3 and sequence analysis**

To clone the TaBln1 and TaCam3 sequences, specific primers (Supplementary Table S1) were designed using the Primer version 5.0 software. The TaBln1 and TaCam3 sequences were PCR-amplified using Suwon 11 cDNA sample as a template. The obtained sequences were aligned using the T. aestivum cv. Chinese Spring (CS) genome from the Ensembl Plants database (http://plants.ensembl.org/Multi/Tools/Blast) and the NCBI (http://www.ncbi.nlm.nih.gov/). The multi-sequence alignments were carried out using DNAMAN version 8 software. The molecular sizes of TaBln1 and TaCam3 were predicted using the Compute pI/Mw tool (https://web.expasy.org/compute_pi/). The signal peptide of TaBln1 was identified using SignalP version 5.0 (http://www.cbs.dtu.dk/services/SignalP/). The transmembrane domain of TaBln1 was identified using TMHMM Server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

**Plasmid construction**

To construct the vector for subcellular localization, the coding sequences for TaBln1 and TaCam3 without a stop codon were amplified and inserted into the NcoI and SpeI restriction sites of the plant binary expression vector pCAMBIA1302 containing the GFP-tag sequence, respectively. In addition, the TaBln1 without a stop codon was inserted into the pCH68988 vector with the mCherry tag. For plasmid constructs for the silencing system, two small cDNA fragments (Supplemental Figure S15) based on the results of a BLASTN search of the NCBI (http://www.ncbi.nlm.nih.gov/) that showed the lowest sequence similarity...
with other wheat genes were inserted into PacI and NotI restriction sites of the virus plasmid, respectively (Holzberg et al., 2002).

For the interaction analyses of TaBln1 and TaCam3 using BiFC, TaBln1 and TaCam3 were subcloned into pSPYNE(R)173 and pSPYCE(M) with BamHI and XhoI restriction sites to generate the pSPYNE(R)173-TaBln1 and pSPYCE(M)-TaCam3 vectors, respectively (Waadt et al., 2008). The coding sequence of TaBln1 was inserted into the SpeI restriction site of pBinGFP2 vector and TaCam3 with HA tag was inserted into the BsaI restriction site of pICH86988 vector to generate pBinGFP2-TaBln1 and pICH86988-TaCam3 for Co-IP. The coding sequences of TaBln1 and TaCam3 were subcloned into the N- or C-terminal fragment of LUC (nLUC or cLUC) with BamHI and SalI restriction sites to generate the TaBln1-nLUC and TaCam3-cLUC vectors for split-LUC assays, respectively. The coding sequence of TaBln1 was subcloned into the EcoRI and XhoI restriction sites of the pGEX-4T-1 expression vector containing the GST-tag sequence; the coding sequence of TaCam3 was subcloned into the EcoRI and XhoI restriction sites of the pET-28a vectors containing the His-tag sequence for the pulldown assay. The primers for all of the plasmid constructions are listed in Supplemental Table S1.

BSMV-mediated gene silencing
To transiently silence TaBln1 and TaCam3, the corresponding vectors (TaPDS-γ, recombinant γ-gene and γ), α and β were linearized using corresponding enzymes and transcribed into RNA. The transcripts of α and β were mixed in a 1:1:1 ratio with transcripts of TaPDS-γ, recombinant γ-gene, and γ in FES buffer (0.1 M glycine pH 8.9, 0.06-M K₂HPO₄, 1% w/v bentonite, 1% w/v sodium pyrophosphate, and 1% w/v celite), respectively. Then the second wheat leaves were inoculated with the BSMV RNA mixture and maintained at 26°C (Holzberg et al., 2002). BSMV:TaPDS (wheat phytoene desaturase) was used as a positive control. When the virus phenotype (photobleaching in positive control) was observed (12 d after BSMV treatment), the fourth leaves were inoculated with Pst CYR23 or CYR31 and samples were harvested at 0, 24, 48, and 120 hpi for histological observation and RNA isolation. RNA was used to confirm the silencing efficiency for each assay by RT-qPCR. The phenotypes were recorded, and representative photographs were captured at 14 dpi. Three independent sets of inoculations were performed, consisting of 60 seedlings inoculated for each BSMV virus.

Histological analyses of silenced plants inoculated with Pst
For the histological observations of fungal growth, the samples of silenced plants were cut into several fragments and decolorized in destaining solution (absolute ethyl alcohol:acetic acid, 1:1 v/v) as previously described (Cheng et al., 2015). Then these fragments were decolorized in chloral hydrate for 24 h followed with autoclaving at 121°C for 2 min.
Wheat germ agglutinin (WGA) conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) was used to stain the Pst infection structures as previously described (Ayliffe et al., 2011).

For the detection of H₂O₂ accumulation, infected leaves were cut and the ends were immersed in a solution containing 1 mg/mL DAB (Amresco) for 4–6 h (Wang et al., 2007). Then these fragments were decolorized in destaining solution to remove the chlorophyll. Only those infected sites where an appressorium had formed over a stoma were considered a successful penetration site of Pst. The accumulation of H₂O₂, hyphal length, infection area, and a number of HBs, HMCs, and haustoria were observed with an Olympus BX-51 microscope (Olympus, Tokyo, Japan) and measured using the cellSens Entry software (Olympus).

**Protein interaction assays**

For the BiFC assay, the pSPYNE(R)173-TaBln1 and pSPYCE(M)-TaCam3 vectors were transformed into *Agrobacterium tumefaciens* GV3101. The *Agrobacterium* strains were co-infiltrated at an OD₆₀₀ of 0.6 into *N. benthamiana* leaves. Two days after inoculation, YFP (Yellow Fluorescent Protein) fluorescence was captured by an Olympus FV3000 Confocal Laser Microscope with a 488-nm laser, 20.0% intensity, 500- to 540-nm collection bandwidth and 491 V gains.

For the Co-IP assay, the pBinGFP2-TaBln1 and pICH86988-TaCam3 vectors were transformed into A. *tumefaciens* GV3101 and co-infiltrated into *N. benthamiana* leaves. The pBinGFP2 empty vector was used as the negative control. Two days after inoculation, the infiltrated leaves were harvested and ground to a powder in liquid nitrogen and then homogenized in RIPA buffer (50-mM Tris–HCl, pH 8, 150-mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, and 0.1% SDS) with 1-mM phenylmethylsulfonyl fluoride (PMSF, Beyotime Biotechnology, Shanghai, China). Then the extract was centrifuged at 12,000 rpm for 10 min, and the supernatant was transferred into a fresh tube for the Co-IP assay. For immunodetection, the supernatant was mixed with 15-μL GFP-trap A beads (Chromotek, Planegg, Germany) and incubated at 4°C for 1.5 h. The beads were collected and washed five times with 500-μL wash buffer (50-mM Tris–HCl, pH 7.4, 150-mM NaCl, and 0.5% Tween-20). Proteins bound to the beads were boiled for 10 min and detected by Western blotting with anti-HA and anti-GFP, respectively. The monoclonal antibodies anti-HA (Beyotime Biotechnology) and anti-GFP (Beyotime Biotechnology) were used at a 1:5,000 dilution and followed by incubation with a second antibody, anti-mouse Ig-horseradish peroxidase (1:2,000; Beyotime Biotechnology). Protein bands were detected using SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific).

Split-LUC assays were performed as previously described (Chen et al., 2008). *A. tumefaciens* GV3101 with different constructs were co-infiltrated into *N. benthamiana* leaves, respectively. After 2 d, 1-mM luciferin (AbMole) was sprayed onto the inoculated leaves, and the LUC activity was captured with a PlantView100 assay system (BLT PHOTON TECHNOLOGY).

For the pulldown assay, GST fusion proteins of TaBln1 and His fusion proteins of TaCam3 were expressed in *Escherichia coli* (BL21) by induction with 0.3-mM IPTG at 16°C overnight. Crude proteins of TaBln1 with GST tag were purified using standard techniques with glutathione-sepharose following a previous study (Harper and Speicher, 2001), and TaCam3 with the His tag was purified via Ni-chelating affinity chromatography. Equal amounts of GST-tagged TaBln1 or GST (negative control) were mixed with TaCam3-His and incubated at 4°C for 2 h with GST beads (Thermo Scientific). The beads were collected and washed five times using the GST Protein Interaction Pull down Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer’s instructions to detect the recovered TaCam3-His levels. The proteins bound to the beads were boiled for 10 min and detected by Western blotting with anti-GST and anti-His antibodies. Monoclonal anti-GST (Beyotime Biotechnology) and anti-His (Beyotime Biotechnology) antibodies were used at a 1:5,000 dilution and followed by incubation with a second antibody, anti-mouse Ig-horseradish peroxidase (Beyotime Biotechnology). The protein bands were detected using SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific).

**Subcellular localization of TaBln1 and TaCam3 in *N. benthamiana***

To determine the subcellular localization of TaBln1 and TaCam3, *Agrobacterium* strains GV3101 harboring TaBln1-GFP and TaWp16-mCherry, a positive control marker protein that is localized to the plasma membrane (Imai et al., 2005), were co-infiltrated into 4-week-old *N. benthamiana* leaves. To determine the co-localization of TaBln1 and TaCam3, *N. benthamiana* leaves were infiltrated with GV3101 carrying TaBln1-mCherry/free mCherry and TaCam3-GFP. The infiltrated seedlings were transferred to a growth chamber at 22°C with a 16-h light photoperiod. GFP fluorescence signals, with a 488-nm laser, 20.0% intensity, 494- to 534-nm collection bandwidth and 338 V gains, and mCherry fluorescence signals, with a 561-nm laser, 20.0% intensity, 570- to 670-nm collection bandwidth and 423 V gains, were monitored at 48-h posttransformation using an Olympus FV3000 Confocal Laser Microscope. All of the assays were performed in duplicate and repeated at least three times.

**RT-qPCR analyses**

Transcription levels were analyzed using a Real-Time PCR Detection System (Bio-Rad Hercules, CA, USA). Primer designs and RT-qPCR reactions were conducted as described previously (Wang et al., 2009b). The wheat elongation factor TaEF-1a gene was used as a wheat internal reference. Fungal biomass changes in the Pst-infected wheat leaves were analyzed as described previously (Chang et al., 2017). Biomass was determined by absolute quantification using a double-standard curve (Lee et al., 2008). The constitutively
expressed wheat stripe rust elongation factor gene \textit{PstEF1} was used for the quantification of wheat stripe rust fungus (Yin et al., 2009). All of the primers used are listed in Supplemental Table S1. RT-qPCR analyses used data from three samples, with each group containing three technical repeats. Relative expression was estimated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Quantification of PAMP-induced gene expression and measurements of net Ca$^{2+}$ flux

The detection of PAMP-induced gene expression was conducted as previously described, with minor modifications (Park et al., 2012; Schoonbeek et al., 2015). Four 2-cm long strips of wheat leaves were cut and placed into 2-mL tubes with water and preinfiltrated by vacuum for 3 \times 1 min. Then the materials were left to recover in a growth cabinet overnight to recover from wounding stress and water infiltration. Afterward, water was replaced by fresh water (mock) or 10-µM flg22 peptide (Bankpeptide) or 10-µM chitin (Santa Cruz Biotechnology Dallas, TX, USA) for 10, 30, 60, or 180 min, followed by freezing in liquid nitrogen. Materials were used for RT-qPCR assays with the primers listed in Supplemental Table S1.

Net Ca$^{2+}$ flux was measured using the NMT-YG-100 (YoungerUSA) as previously described (Ma et al., 2015; Wang et al., 2019). In brief, leaves sampled from transiently silenced seedling were immobilized in measuring buffer (0.1-mM KCl, 0.1-mM CaCl$_2$, 0.1-mM MgCl$_2$, 0.5-mM NaCl, 0.3-mM MES, 0.2-mM Na$_2$SO$_4$, and pH 6.0) for 30-min equilibration. The steady-state fluxes in leaf mesophyll cells were continuously recorded for 5 min prior to the chitin treatments. Thereafter, the chitin was slowly added to the measuring buffer until the chitin concentration reached 10 µM. Then the transient flux of Ca$^{2+}$ was recorded and continued for 10 min.

Data analyses

The data were analyzed using Student’s t test, with GraphPad Prism version 8.0 statistical software to determine the significant differences between control and treatment.

Accession numbers

Sequence data from this article can be found in the National Center for Biotechnology database (http://www.ncbi.nlm.nih.gov/) and the Ensembl Plants portal (http://plants.ensembl.org/Triticum_aestivum/Info/Index) under the following accession numbers: \textit{TaBln1-4A} (AK333112.1), \textit{TaBln1-SB} (OM803180), \textit{TaBln1-SD} (OM803181), \textit{TaCaM3-2A} (TraesCS2A02G098100), \textit{TaCaM3-2B} (TraesCS2B02G113800), \textit{TaCaM3-2D} (TraesCS2D02G097500), \textit{TaCaM3-4A} (TraesCS4A02G126700), \textit{TaCaM3-4B} (TraesCS4B02G178200), \textit{TaCaM3-4D} (TraesCS4D02G179800), \textit{TaEF-1a} (Q03033), \textit{TaPR1} (AF384143), \textit{TaPR2} (DQ090946), and \textit{TaPRS} (FG618781).

Supplemental data

The following materials are available in the online version of this article.

- **Supplemental Figure S1.** Sequence alignment of the three copies of \textit{TaBln1}.
- **Supplemental Figure S2.** Standard curves generated for the absolute quantification of genomic DNA of \textit{Pst} and wheat.
- **Supplemental Figure S3.** Increased H$_2$O$_2$ accumulation in \textit{TaBln1}-silenced leaves after inoculation with the \textit{Pst} CYR31.
- **Supplemental Figure S4.** Sequence alignment of \textit{TaBln1} and \textit{TaBln1Δ} (mutations in the conserved cysteines).
- **Supplemental Figure S5.** The bioinformatics analysis of \textit{TaBln1} protein.
- **Supplemental Figure S6.** Subcellular localization of \textit{TaCaM3} in \textit{N. benthamiana} leaves.
- **Supplemental Figure S7.** Nucleotide sequence alignment of the six copies of \textit{TaCaM3}.
- **Supplemental Figure S8.** Amino acid sequence alignment of the six copies of \textit{TaCaM3}.
- **Supplemental Figure S9.** Transcript profiles of \textit{TaCaM3} in wheat leaves in response to \textit{Pst} infection.
- **Supplemental Figure S10.** Silencing efficiency of \textit{TaCaM3} in wheat leaves of the control (BSMV:γ) and \textit{TaCaM3}-silenced plants.
- **Supplemental Figure S11.** Amino acid alignment of \textit{TaBln1} with \textit{BLN1} and \textit{BLN2} in barley.
- **Supplemental Figure S12.** Expression profiles of \textit{TaCaM3} and \textit{TaBln1} in silenced plants.
- **Supplemental Figure S13.** Subcellular localization of \textit{TaBln1} in \textit{N. benthamiana} leaves with plasmolysis.
- **Supplemental Figure S14.** Subcellular localization of HvBLN1 and mutations of \textit{Tabln1} in \textit{N. benthamiana} leaves.
- **Supplemental Figure S15.** Schematic view of the VIGS construct.

**Supplementary Table S1.** Primers for this research.

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Conflict of interest statement. The authors declare that they have no conflicts of interest.
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