Functional Characterization of Calcineurin Homologs
PsCNA1/PsCNB1 in Puccinia striiformis f. sp. tritici Using a Host-Induced RNAi System

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Abstract

Calcineurin plays a key role in morphogenesis, pathogenesis and drug resistance in most fungi. However, the function of calcineurin genes in Puccinia striiformis f. sp. tritici (Pst) is unclear. We identified and characterized the calcineurin genes PsCNA1 and PsCNB1 in Pst. Phylogenetic analyses indicate that PsCNA1 and PsCNB1 form a calcium/calmodulin regulated protein phosphatase belonging to the calcineurin heterodimers composed of subunits A and B. Quantitative RT-PCR analyses revealed that both PsCNA1 and PsCNB1 expression reached their maximum in the stage of haustorium formation, which is one day after inoculation. Using barely stripe mosaic virus (BSMV) as a transient expression vector in wheat, the expression of PsCNA1 and PsCNB1 in Pst was suppressed, leading to slower extension of fungal hyphae and reduced production of urediospores. The immune-suppressive drugs cyclosporin A and FK506 markedly reduced the germination rates of urediospores, and when germination did occur, more than two germtubes were produced. These results suggest that the calcineurin signaling pathway participates in stripe rust morphogenetic differentiation, especially the formation of haustoria during the early stage of infection and during the production of urediospores. Therefore PsCNA1 and PsCNB1 can be considered important pathogenicity genes involved in the wheat-Pst interaction.

Introduction

Calcineurin, a serine-threonine-specific calcium/calmodulin-dependent protein phosphatase with two subunits (CNA and CNB), regulates a variety of physiological processes, such as growth, morphogenesis, pathogenicity, and membrane stress responses through the calcium signaling pathway in eukaryotes [1,2]. The first fungal calcineurin genes were reported in 1991 from the budding yeast Saccharomyces cerevisiae [3,4] and the filamentous fungus Neurospora crassa [5]. Many homologs of CNA or/cNB have been found in medicinal fungi [6] and plant pathogens such as Botrytis cinerea [7] and Magnaporthe oryzae [8,9]. Recent studies have confirmed that calcineurin controls virulence, hyphal elongation and multiple stress responses in the human pathogens Candida dubliniensis [10], Cryptococcus neoformans [11,12], Candida albicans [13] and Aspergillus fumigatus [14,15]. Similar findings have also been reported for the phytopathogens Ustilago maydis [16], Cochliobolus mayaebeanus [17], and Sclerotinia sclerotiorum [18]. The calcineurin pathway also plays a role in drug resistance to azoles in C. albicans [19,20], and in C. dubliniensis [10]. Inhibition of calcineurin can decrease fungal growth and arrest tissue invasion [21]. This opens possibilities to develop new antifungal agents targeting the calcineurin pathway in fungi [6].

RNA induced gene silencing or RNA interference (RNAi) is a complex natural phenomenon and a powerful reverse genetics tool for the analysis of gene function in eukaryotes [22–26]. In plants, virus-induced gene silencing (VIGS) was developed for rapid functional analysis of plant genes using viruses to deliver silencing constructs [27–29]. It has widely been applied in dicots such as Arabidopsis [30], tobacco [31] and tomato [32–33], and monocots such as barley [34] and wheat [35–38]. In fungi, RNAi technology has been deployed in more than 40 species including plant and human pathogens [39]. Nguyen et al. [40] developed a high-throughput RNA-silencing vector for M. oryzae to identify an involvement of calcineurin genes in colony pigmentation, sporulation, appressorium formation, and pathogenicity. However, for there is still no applicable transformation system available there are currently no techniques on hand for silencing genes in obligate biotrophic fungi directly. Host-induced gene silencing (HIGS) is a newly developed RNAi technology to indirectly silence parasite genes by expressing an RNAi construct in vivo in the host [41]. Host induced RNAi of three target genes suppressed their expression in the plant hopper Nilaparvata lugens after feeding on rice plants [42]. Recent studies confirm the hypothesis that fungal genes can be suppressed in planta during interaction of the fungus...
Characterization of Calcineurin Homologs in _Pst_

Suppressors block _Pst_ germination

The immuno-suppressants cyclosporin A (CsA) and FK506 inhibit calcineurin activity and affect its function just like mutants of CNA or and CNB in several fungi [6]. In order to test whether these two drugs affect the function of _PsCNA1_ and _PsCNB1_, stripe rust urediospores were incubated with these drugs and germination was monitored. After 10 hours, microscopic analyses indicated that germination rate was reduced to 40.5% for FK506 (3 µM) and 66.5% for CsA (3 µM) treatment, compared to water (Table 1). Germ tubes of _Pst_ were limited in their elongation by treatment with of FK506 (1 µM) or CsA (0.1 µM) compared to the control (Fig. 2C, 2B, 2A). Urediospores also frequently produced two or three more irregular germ tubes than the control (Fig. 2D, 2E, 2F).

Expression profiles of _PsCNA1_ and _PsCNB1_

To gain insight into the possible function of _PsCNA1_ and _PsCNB1_ in _Pst_, we investigated the expression of _PsCNA1_ and _PsCNB1_ (mRNA abundance) in different stages of _Pst_ using quantitative PCR (qRT-PCR). _PsCNA1_ and _PsCNB1_ had similar expression profiles (Fig. 3). Transcript levels of both genes were drastically increased at 1.0 dpi, but quickly decreased to 3-fold, and 0.5-fold, respectively, at 11 dpi (Fig. 3). The maximum accumulation of transcript was 58 fold for _PsCNA1_ and 38 fold for _PsCNB1_. However, kinetics of transcript accumulation differed between the two genes. The transcripts for _PsCNB1_ drastically increased over time up to 1 dpi, whereas transcript level of _PsCNA1_ only showed a dramatic increase at 1 dpi.

HIGS for _PsCNA1_ and _PsCNB1_

In order to determine the best inoculation day for _Pst_ inoculation, virus symptoms were scored by visual assessment at four time point (3, 10, 12, 14 dpi) for the BSMV:γ:0-as vector (data not shown). Only three out of eighteen seedlings showed slight virus symptoms at 8 dpi. Symptoms increased at 10 dpi, and almost all seedlings showed 100% virus infection at 12 dpi. At 14 dpi leaves showed heavy symptoms with large yellow areas. Therefore, 12 dpi with BSMV was chosen for rust inoculation.

In order to identify HIGS efficiency (knockdown rates), transcript levels of _PsCNA1_ and _PsCNB1_ were scored in inoculated silenced plants at 8 dpi by qRT-PCR. Results showed that silencing was detected for both BSMV vectors, _PsCNA1_ transcript level exhibited an average of 24% expression in BSMV:γ:_PsCNA1_-as infected plants. However, _PsCNB1_ transcript level showed only an average of 18% reduction. HIGS for both genes lost most effectiveness at 16 dpi (_PsCNA1_: 49% expression, _PsCNB1_: 110% expression) (Fig. 4).

Silencing calcineurin blocks _Pst_ growth and development in wheat leaves

To determine cytological changes associated with fungal growth on plants, silenced for _PsCNA1_ or _PsCNB1_ wheat leaves inoculated with race CYR31 were examined microscopically. Two time points (2 and 5 dpi) were compared. No significant differences in _Pst_ development and hyphal growth were observed between control plants and plants carrying _PsCNA1_ or _PsCNB1_ knock-down constructs at 2 dpi (Table 2; Fig. 5A, 5B, 5C). However, at 5 dpi hyphal length on average in BSMV:γ::_PsCNA1_-as and BSMV:γ::_PsCNB1_-as infected wheat leaves were much shorter than those observed in controls (Table 2).

Results

_PsCNA1_ and _PsCNB1_ encode calcineurin homologs

One of the expressed sequence tags (ESTs) from a full-length cdNA library of _Pst_ [52] was found to be highly similar to _PsCNA1_ from _Puccinia striiformis_ (PTTG_07903) and _PsCNB1_ from _Puccinia graminis_ f. sp. _tritici_ (PGTG_14891). Another two ESTs from _Pst_ cDNA libraries contracted by Zhang et al. [49] and Ma et al. [53] are almost identical to the _CNB_ genes from the other two wheat rusts (PTTG_02210 and PGTG_04308). Further sequencing of these clones from the Chinese _Pst_ race CYR31, provided full-length _cdNA_ sequences of _PsCNA1_ and _PsCNB1_ (Genbank accession numbers, JX424819 and JX424820, respectively). The full length _cdNA_ sequence for _PsCNA1_ is 2.680 bp with an open reading frame (ORF) of 2,097 bp encoding a 698 amino acid (AA) protein, which consists of two calcineurin A domains and six Serine/threonine-protein phosphatase domains with a calculated molecular mass of 76.7 kDa (Fig. S1). The _PsCNB1_ _cdNA_ is 770 bp in length with an ORF of 528 bp encoding a 175 AA protein, with a molecular mass of 19.77 kDa, which has four calcium-binding EF-hand motifs and a N-myristoylation site (Fig. S2).

The levels of conservation of _PsCNA1_ and _PsCNB1_ are indicated in comparison with homologs from other fungi and some model organisms in Figure S1 and Figure S2, respectively. _PsCNA1_ is 90% identical to the calcineurin A subunit of _P. triticina_ and 84% identical to _P. graminis_ f. sp. _tritici_ CNA. _PsCNB1_ is conserved among other fungi analyzed with 54% to 75%. _PsCNA1_ exhibits strong similarity to calcineurin B proteins from other organisms. It is 100% identical to _PcCNB1, 99% identical to _PsCNB1, and 60% to 83% identical to _CNB_ genes from other fungi analyzed.

Phylogenetic analysis revealed that _PsCNA1_ and _PsCNB1_ cluster with other Basidiomycete fungi. Especially the three _Puccinia_ sp. were most close to each other (Fig. 1). However, _PsCNA1_ was closer to _P. graminis_ f. sp. _tritici_ than to _P. triticina_ while _PsCNB1_ was closer to _P. triticina_ than to _P. graminis_ f. sp. _tritici_.

with the host. Tinoco et al. [43] silenced the reporter gene GUS in _Fusarium verticillioides_ by expressing GUS dsRNA in tobacco. HIGS was also successfully used in obligate biotrophic fungi. Using a BSMV-VIGS system expressing the target dsRNA in wheat, Nowara et al. [44] showed that the fungal genes _GTFl_ and _GTF2_ in _Blumeria graminis_ play a role in haustorium formation and elongation of secondary hyphae. Yin et al. [45] also developed a BSMV-based HIGS approach to identify gene function in the biotrophic rust fungus _Puccinia striiformis_ f. sp. _tritici_ ( _PsGr_).

Wheat stripe rust, caused by the Basidiomycete _Pst_, is an important disease in wheat worldwide. As an obligate biotrophic pathogen infecting wheat leaves, _Pst_ undergoes a high degree of morphological and physiological differentiation from urediospore to germ tube, invasive hypha and haustorium, a special structure for nutrient uptake from the host [46–40]. A few studies reported that the calcium signaling pathway is involved in the initial infection and biotrophic growth of rust fungi [49,50]. Some studies showed that the calcium signaling pathway is involved in the initial infection and biotrophic growth of rust fungi [49,50]. Some studies reported that the calcium signaling pathway is involved in the initial infection and biotrophic growth of rust fungi [49,50]. Some studies reported that the calcium signaling pathway is involved in the initial infection and biotrophic growth of rust fungi [49,50]. Some...
Reduction in rust sporulation after silencing Pst calcinurin

We also scored sporulation and found that the number of uredia was reduced on silencing plants (Fig. 6). Sporulation of Pst on silencing plants occurred two days later (12 dpi) than on the control plants (10 dpi). Statistical analyses determined an average number of 99 uredia for control plants, 60 uredia for BSMV:γ:PsCNA1-as, and 68 uredia for BSMV:γ:PsCNB1-as (Table 2). Uredia in silencing plants were smaller in size with open cavities that were shorter, and contained fewer spores (Fig. 7).

Discussion

In this study we describe the isolation and characterization of two calcineurin genes from the wheat stripe rust fungus Pst. Phylogenetic analyses of eukaryotic CNA and CNB genes clearly show that PsCNA1 and PsCNB1 are closely related to calcineurin genes from other basidiomycetes. The calcineurin A/B protein family appears to be conserved in size and structure. Most CNA genes encode a protein of more than 500 amino acids and most CNB proteins contain about 175 amino acids [54]. All CNA proteins share high homology within the N-terminal 50 to 420 amino acid residues, but with significant variations in the C-terminus.

Table 1. Germination rates of Pst (mean ± SE).

| Treatments | Percent Germination (10 hours) |
|------------|--------------------------------|
| Water      | 98.7 ± 0.2%                  |
| FK506 (3 μM)| 40.5 ± 4.7%                  |
| CsA (3 μM) | 66.5 ± 3.1%                  |

Values are significantly different at P = 0.05 according to the Tukey’s test.

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terminal part. With a size of almost 700 aa PsCNA1 is considerably larger than other CNA proteins (Fig. S1). In contrast, PsCNB1 shares length, four conserved EF-hands, and a N-myristoylation site with other CNB proteins (Fig. S2). Especially the three Puccinia sp. have conserved amino acid residues at the N-myristoylation site and the first three EF motifs. Juuvadi et al. [15] found that CnaB is required for localization of CnaA to the septum and that the two calcineurin subunits are required to control hyphal growth and septation in A. fumigatus. PsCNB1 is upregulated earlier than PsCNA1, which might indicate that expression of PsCNB1 is necessary for expression or stability of PsCNA1. Whether PsCNB1 is indeed necessary for stabilization of PsCNA1 and whether the two gene products co-operate still needs more research.

Harel et al. [18] reported that the transcript level of CNA in S. sclerotiorum is 2.5-fold higher in sclerotia than in infection hyphae. In contrast, our results from qRT-PCR show that transcript levels of PsCNA1 and PsCNB1 were much higher at 1 dpi, which corresponds to an early infection stage such as invasive hypha and initial haustorium formation. These differences in expression may be due to the fact that RNAs from S. sclerotiorum were prepared from mycelium grown on artificial medium, while RNAs of Pst were prepared from infected host plants (except germinatedurediospores). Another reason may be different functions of calcineurin in the basidiomycete Pst and the ascomycete S. sclerotiorum.

Complexes of CsA with Cyclophilin A or/and FK506 with FK506-binding protein 12 (FKBP12) interfere with calcineurin binding to other phosphoprotein substrates in eukaryotes [1,6]. The two inhibitors have been applied in numerous fungi to illustrate functions of calcineurin. CsA (10 μg/ml) induced cell-division patterns indistinguishable from mutants in calcineurin in U. maydis [16], or mutants of calcineurin in Ustilago hordei [56] or A. fumigatus [15].

Host induced gene silencing (HIGS) has succeeded to identify functions of parasite genes as an efficient reverse genetic tool in several fungi, insects and nematodes [41]. HIGS has emerged as parasite-derived resistance (PDR) to develop durable resistance in agricultural industry [41]. Virus induced gene silencing (VIGS) mediated by the barley stripe mosaic virus (BSMV) has been successfully developed in wheat [35,36], and recently applied to HIGS with B. graminis [44] and Pst [45]. The BSMV-VIGS system is developed to express double stranded RNA (dsRNA) of targets from Blumeria and Puccinia genes in plants to trigger RNA silencing. We use BSMV-VIGS as viral vector to deliver Pst silencing constructs for calcineurin genes in order to silence them in Pst through the host. The suppression was almost 76% for PsCNA1, but only 18% for PsCNB1 at 8 dpi (Fig. 4). Effective silencing was evident by the reduced number of uredia (Table 2. and Fig. 6), and smaller uredia with less open areas (Fig. 7). Our results illustrate different knock-down efficiencies for Pst HIGS by BSMV vectors with the two calcineurin genes. Silencing efficiency is variable for different genes or even the same gene. This has been shown for thirty-seven genes in M. oryzae [40], eleven genes of Pst [45], inf1 in Phytophthora infestans [57] and GUS in F. verticillioides [43]. However, the phenotype of PsCNB1 was similar to that of PsCNA1 knock downs. An explanation might be that PsCNA1 or/and PsCNB1 act

![Figure 3. Transcript levels of PsCNA1 and PsCNB1 during Pst differentiation stages.](image1)

![Figure 4. Transcript levels of PsCNA1 and PsCNB1 after HIGS during Pst differentiation stages.](image2)

![Figure 5. Histological observation of Pst growth using calcofluor staining.](image3)
The silencing signal can be propagated to the offspring in plants and fungi. VIGS by BSMV vectors can be transferred to the next generation in wheat and barley [38]. Other VIGS vectors still also transmit silencing signals to next generation seedlings. Gene silencing via VIGS-ALSv (Apple Latent Spherical Virus) is from 33% of first progeny seedlings to 53% of subsequent progeny in soybean [27]. Silencing gfp was maintained in subsequent generations of Moniliophthora perniciosa [59]. We did not directly show BSMV heredity to next generation Pst unediospores. But after HIGS to PsCNA1 and PsCNB1, we observed that the new unediospores also had similar defects with strange branched tubes (Fig. 8B, 8C) to that by the immuno-suppressors FK506 and CsA (Fig. 2E, 2F). These results illustrated that PsCNA1 and PsCNB1 take important function in the morphodifferentiation, hyphal development and sporulation in wheat stripe rust.

Although HIGS can be a valuable tool in identifying functions of fungal genes, the question is how the transfer of the silencing signals takes place. Fungal haustoria and the extrahaustorial matrix between host and fungus are specialized places to exchange

### Table 2. Histological observation during HIGS (mean ± SE).

| Treatments | Number of hyphal branches at 2 dpi | Number of haustoria at 2 dpi | Hyphal length at 5 dpi (μm) | Number of uredia at 16 dpi |
|------------|-----------------------------------|-----------------------------|-----------------------------|---------------------------|
| BSMV::γ:0-as | 2.00 ± 0.28                       | 3.21 ± 0.32                 | 283.94 ± 10.41             | 99 ± 7                    |
| BSMV::γ PsCNA1-as | 1.86 ± 0.30                       | 2.62 ± 0.35                 | 183.11 ± 12.78             | 60 ± 8                    |
| BSMV::γ PsCNB1-as | 1.82 ± 0.17                       | 2.59 ± 0.17                 | 197.41 ± 13.09             | 68 ± 5                    |

Abbreviations: dpi, day post inoculation; SE, Standard Error.

1 Leaves infected inoculated with BSMV::γ:0-as (empty vector), BSMV::γ PsCNA1-as and BSMV::γ PsCNB1-as followed by inoculation with CYR31.

2 Distance from the base of the substomatal vesicles to the hyphal tips.

3, 4 Values are not significantly different at P = 0.05 according to the Tukey’s test.

Figure 6. Uredia on silenced leaves 16 days after Pst inoculation. Pst development on wheat leaves after HIGS. A: No virus and Pst (healthy leaf control); B: No virus (normal infection with Pst); C: BSMV::γ:0-as (empty viral vector control); D: BSMV::γ PsCNA1-as (Pst infection after silencing PsCNA1); E: BSMV::γ PsCNB1-as (Pst infection after silencing PsCNB1).

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Figure 7. SEM photograph of uredia after HIGS at 16 dpi. Sorus of silenced PsCNA1/PsCNB1 of Pst infected leaves inoculated with CYR31. Scanning electron micrographs by 600×, scale bar: 20 μm. A: BSMV::γ:0-as (empty viral vector control); B: BSMV::γ PsCNA1-as (silencing PsCNA1); C: BSMV::γ PsCNB1-as (silencing PsCNB1).

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Figure 8. SEM photograph of germinated Pst unediospores after HIGS at 16 dpi. New unediospores of silenced PsCNA1/PsCNB1 of Pst CYR31 in wheat leaves after 6 hour mist moisture. SEM micrographs for A: BSMV::γ:0-as (empty viral vector control), 1500×; B: BSMV::γ PsCNA1-as (silencing PsCNA1), 2000×; C: BSMV::γ PsCNB1-as (silencing PsCNB1), 2000×. Scale bars: 10 μm.

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nutrients and information [47,48]. Nowara et al. [44] regarded the exosomal pathway as the means of secreted dsRNA or siRNA transfer from the host plant wheat into B. graminis. By this way multivesicular bodies and exosomes transport RNAs to the extrahaustorial matrix. Yin et al [45] postulated that RNA silencing signals also extend from the expressing host cell into haustoria of Ps. Another possibility would be that the VIGS-BSMV vectors can cross the extrahaustorial matrix into haustoria in Ps. In this case the rust fungus needs a complete silencing machinery to accomplish degradation of RNAs. Although Argonaute-like proteins (AGO) have been identified in Ps (GenBank accession: AEM611140.1) [51], future work could answer these questions.

Materials and Methods

Strains and RNA isolation

Chinese Ps race CYR31 was inoculated and propagated on wheat cultivar Suwon 11 as described previously [53]. For isolating RNA from infected plants, infected wheat leaves were harvested at 0, 0.5, 1, 3, 7, 9 and 11 days post inoculation (dpi). All material was frozen in liquid nitrogen and stored at −80°C.

Total RNA was extracted using the Qiagen Plant RNeasy kit (Qiagen, Hilden, Germany) from sampled urediospores, germinated urediospores, and infected wheat leaves. First-strand cDNA was synthesized from 1 μg total RNA of each sample using the SMART™cDNA synthesis kit (Clontech Laboratories, Inc., Mountain View, CA) according to the manufacturer’s instructions.

Isolation and sequence analysis of PsCNA1 and PsCNB1

Screening the Ps cDNA library constructed by Ling et al. [52], one EST clone (480 bp) was found to be homologous to CNA genes. Another two EST clones (zyh1090 and mjb959; GenBank accession: ES222865 and GR305110, respectively) with homology to CNB genes were obtained from a Ps cDNA library constructed by Zhang et al. [49] and a cDNA library from wheat leaves inoculated with Ps constructed by Ma et al. [53]. Two primers PsCNA-S/AS and PsCNB-S/AS (Table 3) were designed to full length cDNA sequence of PsCNA1 and PsCNB1 from Ps. The clones of PsCNA1 and PsCNB1 were sequenced on an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Sequences were analyzed using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), BLAST searches against the database of P. graminis f. sp. tritici and P. triticina (http://www.broadinstitute.org/annotation/genome/puccinia_group/GenomesIndex.html), and ORF Finder (http://www.ncbi.nlm.nih.gov/orf/). The alignments of the deduced protein sequences and phylogenetic trees were computed using MEGA4 and ClustalX version 1.83 as described by Guo et al. [60]. PsCNA1 and PsCNB1 sequences have been deposited in GenBank (GenBank accession number JX424819 and JX424820, respectively).

Quantitative RT-PCR

To analyze the transcript levels of the two subunits of Ps calcineurin, relative quantification of gene expression was performed using quantitative RT-PCR (qRT-PCR) on an ABI prism 7500 sequence detection system (Applied Biosystems, Carlsbad, CA). Transcript abundance was assessed with three independent biological replicates. Amplification was performed as follows: 95°C for 1 min, followed by 40 cycles of 10 s at 95°C, 20 s at 60°C and 40 s at 72°C. This was followed by melting curve analysis. The transcript levels of PsCNA1 and PsCNB1 were calculated by the 2^{-ΔΔCt} method with the Ef1 gene of Ps as endogenous reference for normalization as described by Guo et al. [60]. The following primers were used for qRT-PCR: PsCNA1 (PsCNA-1RT S/AS, Table 3), PsCNB1 (PsCNB-1RT S/AS, Table 3). Relative quantification of PsCNA1 and PsCNB1 was computed for the different stages in comparison to that at zero hour inoculation with Ps urediospores.

Construction of BSMV-based VIGS vectors and VIGS assay

BSMV-VIGS vectors are based on the constructs by Holzberg et al. [35]. To avoid non-specific silencing of wheat genes, the target regions of VIGS vectors were blasted for homologs to all wheat sequences in the NCBI database and designed to be rust-specific. Selected PsCNA1 and PsCNB1 gene fragments were amplified by PCR from Ps cDNA using primers with restriction enzymes NotI and PacI sites (Primers: PsCNA-1VS/AS and PsCNB-1VS/AS, respectively; Table 3). Amplicons were ligated into the BSMV γ vector generating BSMV:γ:PsCNA1-as and BSMV:γ:PsCNB1-as. The native BSMV:γ:0-as was used as negative control.

Two-leaf wheat seedlings were used for virus inoculation by rubbing the first leaf as described by Yin et al [43]. Seedlings were incubated in the greenhouse after spraying with water (25°C for 16 hours light and 20°C for 8 hours dark). For inoculation with Ps urediospores, plants were incubated at 20°C for 16 hours light and 16°C for 8 hours dark. Primers (PsCNA-1RT S/AS and PsCNB-2RT S/AS, respectively; Table 3) were used for assaying the transcript levels of PsCNA1 and PsCNB1. Control seedlings were infected with the BSMV:γ:0-as vector and also inoculated with Ps. Total RNA was extracted from leaves of 18 wheat seedlings at two time points (8 and 16 days) after rust inoculation.

Histological observation of Ps growth in wheat leaves

Wheat leaves were sampled at 2 and 5 days and stained with Calcofluor White. The infected leaves were examined with the microscope (Olympus BX-51) to observe Ps haustoria and infection hyphae under UV light. Wheat leaves infected Ps (16 dpi) were observed by scanning electron microscopy (JEM1230).

Table 3. Primers used in this study.

| Primers | Sequence (5’ to 3’) |
|---------|---------------------|
| PsCNA-S | ATGGTAGCCCGCTCCACGAGT |
| PsCNA-AS| TTTGTTGACCGACGCAAGT |
| PsCNB-S | ATGGTGCAACCGGATCACAAC |
| PsCNB-AS| CTAAATAGCCCCTGAGTTC |
| PsCNA-1VS| ATATTATTAAACAACGAGACGAGAA |
| PsCNB-1VS| TGGCGCGCCCGCTGACATGAGTACGG |
| PsCNA-1RTS| CTCTAAGGCCTCAACCGT |
| PsCNB-1RTS| CGGCCAGCAGCAGGAGT |
| PsCNB-1RTS| TGATAGAAGATGGAGGAGAAC |
| PsCNB-2RTS| GTCTCAACGATCGTCTGAGT |
| PsActin-1RTS| TGGATTCTGAGATGGTGT |
| PsActin-1RTAS| CTCTCGGGCCTGTATGGA |
| PsEF-1RTS| TCCGGCTTCCTGATATGAGAAC |
| PsEF-1RTAS| ATCGCATATCGGTGCTGAGT |

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Inhibitor assays using CsA or FK506

CsA (Sigma, USA) was diluted to be 1 mM mother solution in 95% ethyl alcohol. FK506 (Sigma, USA) was diluted to be 1 mM mother solution in DMSO. CsA and FK506 were added to 3 ml of sterile water with P. eriodospores to reach final concentrations of 3 μM and 0.1 μM for CsA, or 3 μM and 1 μM for FK506. Spore suspensions were incubated at 4°C for germination with sterile water-incubated uredospores as control. Germination was examined under the microscope after 10 hours.

Supporting Information

Figure S1 Comparison of PsCNA1 to other homologous CNB proteins. CoCNA (Cryptococcus neoformans var. grubii, AAB97372), PsCNA (Puccinia graminis tritici, EFP89050), PcCNA (P. striiformis f. sp. tritici, JX424191), PsCNA (P. triticina, PTG_07903), ScCNA (Sclerotinia sclerotiorum, XP_001597594), UmCNA (Ustilago maydis, AAP49999). The solid arrow lines show the STPHPHTASE (Serine/threonine-protein phosphatase domains) domains and the dashed line shows the Cacineurin A Domain. Shaded regions indicate the same AA.

Figure S2 Comparison of PsCNB1 to other homologous CNB proteins. BcCNB (Botrytis fuckeliana, XP_001553369), CsCNB (Cryptococcus neoformans var. neoformans, GP014607), MoCNB (Magnaporthe oryzae, ADD104607), NcCNB (Neurospora crassa, CAA73345), BcCNB, (Puccinia graminis tritici, EFP76352), PsCNB (P. striiformis f. sp. tritici, JX424920), PsCNB (P. triticina, PTTG_02210), ScCNB (S. sclerotiorum, SCRG_030830). The first solid arrow line show Myristylation site, the other solid arrow lines show the EF-hands motifs. Shaded regions indicate the same AA.

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Conceived and designed the experiments: ZH JG RTV ZSK. Performed the experiments: ZH JSZ YHD. Analyzed the data: ZH YHYL JG RTV ZSK. Contributed reagents/materials/analysis tools: ZH JSZ YHD. Wrote the paper: ZH JG RTV ZSK.
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