Identification of Pepper CaSBP08 Gene in Defense Response Against Phytophthora capsici Infection

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Little information is available on the role of Squamosa promoter binding protein (SBP)-box genes in pepper plants. This family of genes is known to have transcription characteristics specific to plants and to regulate plant growth, development, stress responses, and signal transduction. To investigate their specific effects in pepper (Capsicum annuum), we screened pepper SBP-box family genes (CaSBP genes) for Phytophthora capsici (P. capsici) resistance genes using virus-induced gene silencing. CaSBP08, CaSBP11, CaSBP12, and CaSBP13, which are associated with plant defense responses against P. capsici, were obtained from among fifteen identified CaSBP genes. The function of CaSBP08 was identified in pepper defense response against P. capsici infection in particular. CaSBP08 protein was localized to the nucleus. Silencing of CaSBP08 enhanced resistance to P. capsici infection. Following P. capsici inoculation, the malondialdehyde content, peroxidase activity, and disease index percentage of the CaSBP08-silenced plants decreased compared to the control. Additionally, the expression levels of other defense-related genes, especially those of CaBPR1 and CaSAR8.2, were more strongly induced in CaSBP08-silenced plants than in the control. However, CaSBP08 overexpression in Nicotiana benthamiana enhanced susceptibility to P. capsici infection. This work provides a foundation for the further research on the role of CaSBP genes in plant defense responses against P. capsici infection.

Keywords: pepper, Phytophthora capsici, SBP-box family genes, CaSBP08, defense genes, Nicotiana benthamiana

INTRODUCTION

Pepper (Capsicum annuum L.) is a valuable solanaceous crop species with high economic as well as high nutritive value worldwide. However, it is prone to various diseases, especially Phytophthora blight, which is caused by Phytophthora capsici (Jin et al., 2016), a soil-borne pathogen that can infect various tissues of pepper plants, including roots, stems, leaves, flowers, and fruits (Wang et al., 2013a). Its host range is wide, including tomato, eggplant, cucumber, watermelon, pumpkin, snap peas, and lima beans (Granke et al., 2012). To combat pathogen infection, plants have developed a range of defense mechanisms including physiological, biochemical, molecular, and cellular processes and barriers along with inducible innate immunity (Mou et al., 2017; Hou et al., 2018). Inducible innate immunity is largely regulated at the transcriptional level by the action of many proteins, including members of the SBP-box family.
transcriptional factors. Transcription factors have important functions in combating the impact of pathogens through activitating or repressing the expression of defense genes (Xu et al., 2011). For example, APETALA2/Ethylene Response Factor-domain transcription factor (AP2/ERF-ORA59) directly regulates the expression of PDF1.2 (PLANT DEFENSIN1.2) in the process of necrotrophic pathogen infection in Arabidopsis (Zarei et al., 2011). The basic leucine zipper (bZIP) of the Arabidopsis TGA6 transcription factor acts together specifically with the translocation of the NONEXPRESSION OF PATHOGENESIS-RELATED GENES1 (NPR1) ankyrin repeat protein, which activates the salicylic acid (SA) marker gene PATHOGENESIS-RELATED-1 (PR-1) in the Botrytis cinerea necrotrophic infection process (Zhang et al., 1999; Zander et al., 2010). JAMYC2 and JAMYC10 are two other MYC transcription factors that are jasmonic acid (JA)-regulated and involved in defense responses against pathogen infection through inducing PDF1.2 gene expression in tomato (Boter et al., 2004; Lorenzo and Solano, 2005).

Squamosa promoter binding protein (SBP)-box genes are a family of plant-specific transcription factors that contain a highly conserved SBP domain (Klein et al., 1996). The domain contains about 76 amino acids residues, including two zinc fingers and one nuclear localization signal (Yamasaki et al., 2004). Klein et al. (1996) first discovered Antirrhinum majus SBP-box genes, identified as AmSBP1 and AmSBP2, according to their capability to interact with the floral meristem identity gene SQUAMOSA promoter sequence. Subsequently, Lännenpää et al. (2004) identified SBP-box genes in another plant, Betula pendula (i.e., BpSBP1). Similarly, Hou et al. (2013) discovered VpSBP5 in Vitis vinifera, and Zhang B. et al. (2017) found two others in hexaploid wheat, i.e., TuSBP20 and TuSBP21. There are many reports of SBP-box gene involvement in the development and growth of plants. However, there are few reports about its involvement in responses to biotic and abiotic stresses, especially the former (Stone et al., 2005; Wang et al., 2016; Hou et al., 2018). For example, AtSPL14 is induced by the fungal toxin fumonisin B1, which induces programmed cell death in Arabidopsis (Stone et al., 2005). Transgenic Arabidopsis JASMONATE CARBOXYL METHYLTRANSFERASE (AtIMT) plants exhibit down-regulation of AtSPL2 (Atg43270), which has a role in the JA-mediated resistance pathway (Kim et al., 2007). Tobacco NbSPL6 is needed to develop N-mediated resistance for combating tobacco mosaic virus. Moreover, the ortholog AtSPL6 Arabidopsis gene is necessary for the Toll and Interleukin-1 Receptor Nucleotide Binding-Leucine Rich Repeat (TIR-NB-LRR) to function in mediating resistance against Pseudomonas syringae infection (Padmanabhan et al., 2013). Hou et al. (2013) reported that through the SA-induced systemic acquired resistance pathway, VpSBP5 participates in regulating resistance against Erysiphe necator and, in grapes, through the methyl jasmonate (MeJA)-induced wound signaling pathway. AtSPL9 interacts with jasmonate ZIM-domain (JAZ) proteins and negatively regulates the JA response as well as resistance to insects in Arabidopsis (Mao et al., 2017). SPL6 functions in the endoplasmic reticulum (ER) stress response to control ER stress signaling outputs and retain equilibrium between both adaptive and death signals to determine cell fates in rice during ER stress (Wang et al., 2018). However, compared with the widespread research on SBP-box genes in model species such as Arabidopsis, less information is available concerning pepper SBP-box genes, especially regarding their involvement in resistance against P. capsici.

In our previous research, we identified fifteen SBP-box genes in pepper (i.e., CaSBP01, CaSBP02, CaSBP04, CaSBP05, CaSBP06, CaSBP07, CaSBP08, CaSBP10, CaSBP11, CaSBP12, CaSBP13, CaSBP14, CaSBP15, C11g004690), and we named them according to their chromosomal order (Zhang et al., 2016). The CaSBP coding sequences ranged from 336 base pair (bp) to 3024 bp in length for CaSBP08 and CaSBP06, respectively, with a nuclear localization signal occurring for all gene family members except CaSBP08. Additionally, all the CaSBP genes encoded proteins with two zinc finger-like structures, i.e. C3H and C2HC, except for CaSBP09 and CaSBP15, which lack the C3H zinc finger-like structures (Zhang et al., 2016). All CaSBP genes are induced by compatible or incompatible strains of P. capsici, except for CaSBP15, whose expression is down-regulated during P. capsici infection. Some CaSBP genes (i.e., CaSBP11 and CaSBP12) may also be involved in SA and MeJA regulation mechanisms (Zhang et al., 2016). To further study the function of CaSBP genes in plant resistance against the pathogen P. capsici, CaSBP08, CaSBP11, CaSBP12, and CaSBP13, which are involved in the plant defense response against P. capsica, were obtained from among the fifteen identified CaSBP genes using virus-induced gene silencing in this work. In addition, we further investigated the function of CaSBP08 and found it to be localized to the nucleus and to play a negative regulatory role in the plant defense response against P. capsici infection in pepper and transgenic Nicotiana benthamiana. This work provides a foundation for further research on the role of pepper SBP-box genes in plant defense responses against P. capsici infection.

**MATERIALS AND METHODS**

**Plant Material and Pathogen Preparation**

Pepper cultivar AA3 and the P. capsici strain HX-9, provided by the Capsicum Research Group, College of Horticulture, Northwest A&F University, P. R. China, were tested. Plants were maintained in a growth chamber at 22°C/18°C (day/night temperatures) with a 16/8-hour photoperiod. The P. capsici strain HX-9 was cultured in darkness at 28°C with potato dextrose agar (PDA) medium. Sporulation induction and spore release were conducted using the method described by Wang et al. (2011) with modifications. Briefly, the HX-9 strain of P. capsici was first cultured on PDA in a Petri dish under darkness at 28°C for five days. Then, ten approximately 0.8-cm diameter discs were cut from PDA culture plates and grown in the dark for 3 days in 90-mm Petri dishes with 15–20 mL of 2%
milliliters of 1 × 10^5 spores/mL zoospore culture of the silenced and control plants. Then, an assay of the detached with 22°C/18°C (day/night temperatures), a 16/8-hour photoperiod, the injected plants were maintained in a growth chamber set at 18°C family according to the method described by Zhang et al. (2013). All stage were used for the procedures silencing the SBP-box genes freeze-thaw transformation. Pepper seedlings at the two true leaves transformed into (phytoene desaturase, positive control), and TRV1, were TRV2: CaSBP14, TRV2: CaSBP07, and TRV2: CaSBP08 (2016). Then, 5 mL of this zoospore culture was used to inoculate N. benthamiana leaves with a needleless syringe (Mou et al., 2017). After being injected, the plants were grown in a chamber set at 22°C/18°C (day/night) temperatures and a 16/8-hour photoperiod for two days and then assessed under a fluorescent confocal microscope (Olympus, Tokyo, Japan) with a 488 nm excitation wavelength.

**Virus-Induced Gene Silencing of SBP-Box Family Genes in Pepper**

The virus-induced gene silencing (VIGS) system based on tobacco rattle virus (TRV) was used to silence the SBP-box family pepper genes, as previously reported by Wang (2013). To generate the VIGS plasmid constructs of CaSBP genes, fifteen 200–500 bp fragments from the corresponding SBP-box genes were amplified using gene-specific primers. Then, their specificities were assessed using NCBI Primer BLAST (Table S1). Using double digestion, the acquired products were cloned into the TRV2 vectors with BamHI and KpnI restriction enzymes (Zhang et al., 2003). Then, they were sequenced by Sangon Biotech Company (Shanghai, China). The recombinant vectors, i.e., TRV2:CaSBP01, TRV2:CaSBP02, TRV2: CaSBP03, TRV2:CaSBP04, TRV2:CaSBP05, TRV2:CaSBP06, TRV2:CaSBP07, TRV2:CaSBP08, TRV2:CaSBP09, TRV2: CaSBP10, TRV2:CaSBP11, TRV2:CaSBP12, TRV2:CaSBP13, TRV2:CaSBP14, TRV2 (negative control), TRV2:CaPDS (phytoene desaturase, positive control), and TRV1, were transformed into Agrobacterium tumefaciens strain GV3101 using freeze-thaw transformation. Pepper seedlings at the two true leaves stage were used for the procedures silencing the SBP-box genes family according to the method described by Zhang et al. (2013). All the injected plants were maintained in a growth chamber set at 18°C under darkness for two days and then moved to a growth chamber with 22°C/18°C (day/night temperatures), a 16/8-hour photoperiod, and 60% relative humidity. Forty-five days after infiltration, the silencing efficiency was measured from leaf samples collected from the silenced and control plants. Then, an assay of the detached leaves was conducted as described by Zhang et al. (2015). Five milliliters of 1 × 10^5 spores/mL zoospore culture of P. capsici strain HX-9 was used to inoculate the control and silenced plants by drenching roots. Lastly, the roots and leaves of control and silenced plants were collected and stored at -80°C.

**Subcellular Localization of CaSBP08**

The CaSBP08 coding region without a termination codon was amplified using sequence-specific primers (Table S1). The obtained product was cloned into the PMD-19 vector and then cloned into the pVBG2307:GFP vector between the XbaI and KpnI restriction sites to produce the final pVBG2307:CaSBP08:GFP plasmid. The recombinant plasmid was sequenced by Sangon Biotech Company and then transformed into A. tumefaciens strain GV3101 through freeze-thaw transformation. Then, the GV3101 cells carrying the pVBG2307:CaSBP08:GFP vector and pVBG2307:GFP vector (used as a control) were grown overnight in Luria-Bertani (LB) medium with the proper antibiotics. Then, infiltration buffer (10 mM MES, pH 5.7, 10 mM MgCl2, and 200 µM acetosyringone) was used for cell suspension, which was infiltrated into N. benthamiana leaves with a needleless syringe (Mou et al., 2017). After being injected, the plants were grown in a chamber set at 22°C/18°C (day/night) and temperatures and a 16/8-hour photoperiod for two days and then assessed under a fluorescent confocal microscope (Olympus, Tokyo, Japan) with a 488 nm excitation wavelength.

**N. benthamiana Transformation**

The full encoding region of CaSBP08 (336 bp) was cloned into the pVBG2307:GFP vector between the XbaI and KpnI restriction sites to produce the final plasmid pVBG2307:CaSBP08:GFP restriction sites for the procedure. Overexpression lines of CaSBP08 were achieved through tobacco leaf disc transformation with Agrobacterium intervention (Oh et al., 2005). Eleven lines of transgenic N. benthamiana plants, each with resistance to kanamycin and having the pVBG2307:CaSBP08:GFP construct were obtained. Three transgenic lines of CaSBP08 (lines 2, 10, and 11) were randomly selected for further study. Transformation was confirmed using quantitative real-time PCR during the T2 generation (Table S2). T1 plants seeds were obtained from T0 regenerated plants, and T2 lines seedlings were generated on MS agar plates with 100 µg/mL kanamycin. For further analyses, T3 plants were used.

**RNA Extraction and Quantitative Real-Time PCR**

Total RNA was isolated as per the procedures described by Guo et al. (2012). The first strand cDNA was synthesized using the PrimeScript Kit (Takara, Dalian, China) according to the manufacturer’s instructions. The cDNA concentration was diluted to 50 ng/µL and used for quantitative real-time PCR (qRT-PCR). Then, qRT-PCR was performed in triplicate on an iCycler IQ™ Multicolor PCR Detection System (Bio-Rad, Hercules, CA, USA) with the following thermal cycling program: pre-denaturation at 95°C for 1 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. All the primer specificities for qRT-PCR were assessed using NCBI Primer BLAST (Table S2). Gene expression was quantified and normalized to the expression level of actin (CaActin2, accession no. AY572427; Nbactin-97, accession No. LOC109206422) (Schmittgen and Livak, 2008; Du et al., 2015; Yin et al., 2015).

**Malondialdehyde Measurement**

Following inoculation with P. capsici, the malondialdehyde (MDA) content of the control plants and CaSBP08-silenced
plants were measured using a colorimetric determination technique with thiobarbituric acid from Ma et al. (2013) with modifications. For this purpose, the crude enzyme used for MDA determination was extracted using 10% trichloroacetic acid (TCA). Then, 2 mL of crude enzyme extract was mixed with 5 mL of 0.5% thiobarbituric acid (TBA) reagent, boiled for 10 min, quickly cooled, and centrifuged at 5000 × g for 10 min. The control contained 2 mL of distilled water instead of the crude enzyme. Absorbance was measured at 600 nm, 532 nm, and 450 nm.

**Peroxidase and Catalase Activity Measurements**

After inoculation with *P. capsici*, the peroxidase (POD) and catalase (CAT) activities of the CaSBP08-silenced and control plants were measured using the guaiacol method and ultraviolet spectrophotometry, respectively (Hammerschmidt et al., 1982). The crude enzymes, used to determine POD and CAT activity, were extracted using 0.2 M phosphate buffer (pH 7.8) and 0.05M Tris–HCl buffer (pH7.0), respectively. The POD activity determination reaction included 0.1 mL of crude enzyme, 2 mL of 0.3% H₂O₂, and 0.9 mL of 0.2% guaiacol. The CAT activity determination reaction included 1.0 mL of Tris-HCl, 1.7 mL of distilled water, 0.5 mL of crude enzyme, and 0.1 mL of 100 µM H₂O₂.

**Disease Index Percentage Statistics**

Subsequent to *P. capsici* inoculation, the percent disease index values of plants were recorded following the procedure described by Zhang (2009). Sixteen days after *P. capsici* HX-9 strain inoculation, the CaSBP08-silenced and control plants infection symptoms were categorized into five levels: Level 0, no symptoms; Level 1, lower leaves of plants yellowing or wilting; Level 2, lower leaves of plants with obvious defoliation or whole plants wilting; Level 3, stem base black, except for new growth, with all leaves fallen; and Level 4, whole plant death. Thirteen days post-inoculation with *P. capsici*, the symptoms in the transgenic lines were also categorized into five levels: Level 0, no symptoms; Level 1, whole plant wilting, with no constriction between stems and leaves; Level 2, whole plant wilting, with death of lower leaves and constriction between stems and leaves; Level 3, all leaves dead, except those at the point of new growth, with constriction occurring between stems and leaves; Level 4: death of the whole plant. Disease index percentages were recorded based on the following formula:

\[
\text{Disease index percentage} = \left( \frac{\text{the numerical grade of disease} \times \text{number of disease plants of this grade}}{\text{the highest grade of disease} \times \text{total number of surveys}} \right) \times 100
\]

**Statistical Analysis**

Least significant difference (LSD) values were calculated using Data Processing System 7.05 (DPS 7.05, China), a software package with comprehensive experimental design and statistical analysis functions. Significance was determined at \( P \leq 0.05 \) or \( P \leq 0.01 \) thresholds. All the experiments, with at least three biological replicates, were conducted and evaluated separately.

**RESULTS**

**Pepper Plant CaSBP08, CaSBP11, CaSBP12, and CaSBP13 Genes Are Involved in Resistance to *P. capsici***

Phenotypic Observation and Silencing Efficiency of CaSBP-Silenced Plants

To screen for genes that respond to *P. capsici* infection, fifteen CaSBP genes were silenced using the VIGS method. In this study, the pepper *CaPDS* gene (phytoene desaturase, GenBank accession number, X68058) was taken as a positive control, which induces a leaf photo-bleaching phenotype when silenced. For the negative control, an empty TRV2:00 vector was selected. Forty days after injection, the positive control (TRV2:CaPDS) plants showed photo-bleaching, while the TRV2:00 plants and plants with each of the fifteen CaSBP genes silenced exhibited no obvious phenotypic changes (Figure 1A). Subsequently, we detected the silencing efficiency of the fifteen CaSBP genes. As shown in Figure 1B, the fifteen CaSBP genes were silenced compared with the negative control, and the silencing efficiencies were between 50% and 90%. To ensure the silencing specificity of the target CaSBP genes, the expression of both the target genes as well as the genes with the highest homology to the target genes were also measured. As shown in Figure 1C, when the target gene was silenced, the expression levels of the CaSBP genes and their respective genes with the highest homology can be divided into three categories. The first category includes silenced target genes (i.e., CaSBP02 or CaSBP09) for which their corresponding gene of highest homology (i.e., CaSBP06 or CaSBP15) is also silenced, but with a silencing efficiency lower than that of the target gene. The second category included target genes that were silenced (i.e., CaSBP04, CaSBP11, CaSBP12, CaSBP14, or CaSBP15), but their corresponding highest homology genes (i.e., CaSBP12, CaSBP09, CaSBP04 or CaSBP09) had increased expression. The third category includes silenced target genes (i.e., CaSBP05 or CaSBP10) with corresponding highest homology genes (i.e., CaSBP10 or CaSBP05) having unchanged expression. The CaSBP genes with the highest homology with the targeted silenced genes are shown in Table S3.

**Identification of Resistance Associated With CaSBP Genes**

Forty-five days after injection, detached leaves of plants silenced for each of the fifteen CaSBP genes and those of the negative control plants were inoculated with *P. capsici* strain HX-9. Three days post-inoculation with HX-9, the detached leaves of the negative control plants exhibited large hygrophanous lesions, which occupied almost 80% of the whole leaf area (Figure 2A). Additionally, the detached leaves of the plants silenced for CaSBP01, CaSBP02, CaSBP03, CaSBP04, CaSBP05, CaSBP06, CaSBP07, CaSBP09, CaSBP10, CaSBP14, and CaSBP15 also
exhibited large hygrophanous lesions without any noticeable difference from the negative control (Figures 2A, B). However, the detached leaves of the CaSBP08-, CaSBP11-, CaSBP12-, and CaSBP13-silenced plants exhibited smaller hygrophanous lesions or lacked them altogether (Figure 2A). In addition, the percentages of lesion areas on the leaves of CaSBP08-, CaSBP11-, CaSBP12-, and CaSBP13-silenced plants and negative control plants also significantly differed (Figure 2B). Detailed statistics of the disease incidence of detached leaves from CaSBP-silenced and negative control plants are available in Table S4. These results indicated that four CaSBP genes (CaSBP08, CaSBP11, CaSBP12, and CaSBP13) responded to P. capsici infection in pepper.

**CaSBP08 Protein Localization in the Nucleus**

To determine the subcellular localization of CaSBP08 protein, the GV3101 strain of *A. tumefaciens* with pVBG2307:CaSBP08:GFP and pVBG2307:GFP (used as a control) vectors were rapidly expressed in the leaves of *N. benthamiana* plants. The results indicated that the control (pVBG2307:GFP) exhibited GFP signals in the whole cell, including the nucleus, cytoplasm, cell wall, and cell membrane, whereas, pVBG2307:CaSBP08:GFP only exhibited GFP signals in the nucleus (Figure 3). This indicated that CaSBP08 protein was localized in the nucleus.

**Silencing of CaSBP08 Enhanced Pepper Resistance to P. capsici Infection**

In order to confirm the CaSBP genes involved in resistance to *P. capsici* infection, we first selected CaSBP08 for further study using the virus-induced silencing procedure. As Figure S1 shows, the CaSBP08 gene was considerably silenced, and the silencing efficiency was 75%. Two days after inoculation with the *P. capsici* HX-9 strain, the detached leaves of negative control plants showed obvious hygrophanous lesions, while those of the CaSBP08-silenced plants displayed no or small hygrophanous lesions (Figure 4A). Additionally, the average diseased area of the leaves of CaSBP08-silenced plants was significantly smaller than that of the negative control plants (Figure 4B). Additionally, sixteen days after *P. capsici* infection, the damage to CaSBP08-silenced plants was less than that to control plants (Figure 4C). The disease index percentages of CaSBP08-silenced plants were substantially lower than those for the control plants (Figure 4D). Moreover, the MDA content in the CaSBP08-silenced plant was lower than that of the control plants (Figure 4E-1). The POD and CAT activities increased in the
CaSBP08-silenced and negative control plants (Figures 4E-2, 4E-3). However, the POD and CAT activities in the CaSBP08-silenced plants were less than those in the negative control plants (Figures 4E-2, 4E-3). The CaSBP08 expression level increased in the beginning and then decreased, but the expression level in the silenced plants was considerably lesser than that in the negative control plants (Figure 5A). The expression of the defense genes (CaDEF1, AF442388; CaSAR8.2, AF112868; CaPO1, AF442386; CaBPR1, AF053343) increased in CaSBP08-silenced plants to different degrees, and the expression level was more than that in the negative control plants at day one (Figures 5B–E). However, the expression of the defense-related genes (i.e., CaPO1, CaDEF1, CaBPR1, and CaSAR8.2) in the CaSBP08-silenced and negative control plants decreased at day two (except for CaDEF1; Figures 5B–E). The results revealed that CaSBP08 played a negative role in the plant defense response against P. capsici infection.

FIGURE 2 | Phenotype and percentage of the lesion area of detached leaves of the fifteen members of CaSBPs silenced and control plants after inoculation with P. capsici. (A) Phenotype of detached leaves of the fifteen members of CaSBPs silenced and control plants after inoculation with P. capsici. Photographs were taken after inoculation with P. capsici three days. The diameter of the plug of P. capsici is 0.5cm. The red dotted line was used to label the lesion area in each leaf. (B) Percentage of the lesion area of the leaves three days after inoculation with P. capsici. Bars with different lower case letters indicate significant differences at P ≤ 0.05. Mean values and SDs for three biological replicates are shown.

Overexpression of CaSBP08 in N. benthamiana Increased Susceptibility to P. capsici Infection

In order to confirm CaSBP08 is involved in plant resistance to P. capsici infection, transgenic CaSBP08 lines were obtained by Agrobacterium-mediated tobacco leaf disc transformation, as the stable transformation of pepper plants remains challenging. Eleven transgenic lines were acquired, and there were no observable differences among their phenotypes. Then, three CaSBP08 transgenic lines (lines 2, 10, and 11) were randomly selected for disease resistance assays. Seedlings of forty-day-old plants were used for the following experiment. Two days after the P. capsici HX-9 strain inoculation, a small hygrophanous lesion area appeared on the detached leaves of wild-type (WT) plants, while the hygrophanous lesion area occupied almost half of the detached leaves from transgenic lines 2, 10, and 11 (Figure 6A). Additionally, the average areas of disease of transgenic lines 2, 10,
and 11 were significantly higher than that of WT plants (Figure 6B). Three days after HX-9 strain inoculation, no disease symptoms were observed in WT plants, whereas in transgenic plants (lines 2, 10, and 11) wilting and constriction at the junction of the root and stem were observed (Figure 6C). In addition, the forty-five-day-old seedlings were used for the disease index percentage statistics experiment. Thirteen days post-inoculation with \textit{P. capsici} strain HX-9, the disease symptoms in WT and transgenic plants (lines 2, 10, and 11) were categorized into five levels (Figure 6D). The disease index percentages of transgenic lines 2, 4, and 11 were substantially higher than that of WT plants (Figure 6E). A detailed summary of disease index percentage data is provided in Table S5.

The \textit{CaSBP08} expression in transgenic lines 2, 10, and 11 differed from that of WT plants (Figure 6F). The expression of defense-related genes, i.e., \textit{NbPR1a} (pathogenesis-related), \textit{NbPR1b} (pathogenesis-related), \textit{NbDEF1} (defensin), and \textit{NbNPR1} (non-expressor pathogenesis-related), were measured. After \textit{P. capsici} inoculation, the expression of \textit{NbPR1a} (JN247448.1) and \textit{NbPR1b} (XM_016587501.1) increased at day two, and its level in transgenic lines was more than that in the WT plants (Figure 6G). The expression levels of \textit{NbDEF1} (X99403) and \textit{NbNPR1} (AF480488) in WT plants increased at day two and were higher than that in the transgenic lines (Figure 6G). However, the expression levels of \textit{NbDEF1} and \textit{NbNPR1} in transgenic lines had no obvious changes between day one and day two. These results reveal that \textit{CaSBP08} has a negative role in the defense response of plants against \textit{P. capsici} infection.

**DISCUSSION**

The SBP-box gene family is comprised only of plant transcription factors. Gene family members participate in different pathways, including those related to plant morphogenesis, floral transition, male sterility, biosynthesis of gibberellic acid (GA), transition from the vegetative to reproductive stage, endoplasmic reticulum (ER) stress signaling, and environmental stress responses (Cardon et al., 1997; Zhang et al., 2007; Shikata et al., 2012; Ning et al., 2017; Zhang et al., 2017). These roles of SBP-box genes were investigated in other plants. However, the function of \textit{CaSBPs}, especially in plant defense responses against \textit{P. capsici} infection, have not yet been studied.

We screened a gene (\textit{CaSBP05}) identified as differentially expressed from our previously established transcriptome database of \textit{P. capsici} across different affinity races. We analyzed the expression patterns of \textit{CaSBP} genes under infections with \textit{P. capsici}-compatible and -incompatible strains and hormonal treatment in our previous study (Zhang et al., 2016). We found that most \textit{CaSBP} genes are induced by hormones and \textit{P. capsici} infection, but there is no direct evidence proving that they are involved in pepper defence mechanisms against \textit{P. capsici} infection (Zhang et al., 2016). Therefore, to screen \textit{P. capsici} infection response genes from among \textit{CaSBP} genes, fifteen \textit{CaSBP} genes were silenced. The phenotypes of the plants with each of the fifteen silenced \textit{CaSBP} genes exhibited no obvious differences compared with the
control plants (Figure 1A). It is known that most of the genes in SBP-box family are related to plant growth, development, and morphogenesis. For example, overexpression of the small RNA molecule miR156/157, whose target is the protein-regulated SBP-box genes \textit{TfLFY} and \textit{TfMIR172} in \textit{Torenia} plants, can induce bushy plant architectures in \textit{Torenia fournieri} (Shikata et al., 2012). Overexpression of tae-miR156, whose target is the squamosa promoter binding protein-like genes (\textit{TaSPL3/17}) in the bread wheat cultivar ‘Kenong199,’ leads to an increase in tiller number and serious flaws in spikelet development, and tae-miR156 mediated suppression of some squamosa promoter binding genes (Liu et al., 2017). \textit{Arabidopsis AtSPL14} mutants with a T-DNA insertion in their squamosa binding protein (SBP) domain exhibited altered architectures with petiole elongation and more serrated leaf margins (Stone et al., 2005). However, these phenomena were not observed in pepper plants with each of the fifteen \textit{CaSBP} genes silenced. Moreover, in order to confirm the silencing specificity of each of the \textit{CaSBP} genes, we also measured the relative expression of genes with the highest homology compared to each of the silent genes.

When the target pepper SBP-box genes were silenced, the expression levels of the pepper SBP-box genes and their corresponding genes with the highest homology can be divided into three categories (Figure 1C). In the first, the target gene was silenced (i.e., \textit{CaSBP02} or \textit{CaSBP09}), while their corresponding genes with highest homology were also silenced, but with a lower silencing efficiency. The second category included target genes that were silenced (i.e., \textit{CaSBP04}, \textit{CaSBP11}, \textit{CaSBP12}, \textit{CaSBP14}, or \textit{CaSBP15}), while their corresponding genes with highest homology (i.e., \textit{CaSBP12},

![FIGURE 4 | Loss function analysis of \textit{CaSBP08} in pepper plant defense response against \textit{P. capsici} infection. (A) Phenotypes of detached leaves of \textit{CaSBP08} silenced and negative control plants after inoculation with \textit{P. capsici}. Photographs were taken at two days after inoculation with \textit{P. capsici}. The yellow dotted line was used to label the lesion area in each leaf. (B) The average diseased areas of the detached leaves of the \textit{CaSBP08} silenced and negative control plants. Data were collected, two days after inoculation with \textit{P. capsici}. (C) Phenotypes of the \textit{CaSBP08} silenced and negative control plants after inoculation with \textit{P. capsici} sixteen days. (D) The disease index percentage of the \textit{CaSBP08} silenced and negative control plants and data were collected sixteen days after inoculation with \textit{P. capsici}. (E) Determination of MDA content (E-1), POD activity (E-2), and CAT activity (E-3) of \textit{CaSBP08} silenced and negative control plants after inoculating with \textit{P. capsici}. Bars in Figure A are 0.5cm, and C are 4.5cm. Bars with different letters indicate significant differences at $P \leq 0.05$. * Represent significant differences at $P \leq 0.05$. Mean values and SDs for three biological replicates are shown.]


CaSBP09, CaSBP04, or CaSBP09) showed increased expression. The third category included target genes that were silenced (i.e., CaSBP05 or CaSBP10), with their genes of highest homology (i.e., CaSBP10 or CaSBP05) remaining unchanged (Figure 1C).

Among the fifteen CaSBP genes, two genes (CaSBP02 and CaSBP06) contained ankyrin repeats that are present in proteins with different biological roles and are involved in interactions between proteins (Zhang et al., 1999). Thus, there may be a functional relationship between the CaSBP genes. The detached leaves of the fifteen different CaSBP-silenced plants and the negative control plants were inoculated with the P. capsici HX-9 strain. After three days, the detached leaves of the CaSBP01-, CaSBP02-, CaSBP03-, CaSBP04-, CaSBP05-, CaSBP06-, CaSBP07-, CaSBP09-, CaSBP10-, CaSBP14-, and CaSBP15-

**FIGURE 5** The expression of CaSBP08 (A), and defense-related genes, CaDEF1 (B), CaSAR8.2 (C), CaP01 (D), and CaBPR1 (E) after inoculation with P. capsici in CaSBP08 silenced and negative control plants. * and ** represent significant differences at P ≤ 0.05 and P ≤ 0.01 respectively. Mean values and SDs for three biological replicates are shown.
silenced plants exhibited large hygrophanous lesions with no difference compared to the negative control (Figure 2). Additionally, the detached leaves of the CaSBP08-, CaSBP11-, CaSBP12-, and CaSBP13-silenced plants exhibited very small or no hygrophanous lesions, and the percentage of lesion area of the plants silenced for these genes exhibited a significant difference compared with the negative control (Figure 2). Therefore, we screened four CaSBP genes (CaSBP08, CaSBP11, CaSBP12, and

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**Figure 6** | Function analysis of overexpression of CaSBP08 transgenic lines in defense response against *P. capsici* infection. (A) Phenotypes of the detached leaves of transgenic and wild-type plants after inoculation with *P. capsici* two days. The red dotted line was used to label the lesion area in each leaf. (B) The average diseased areas of the detached leaves of transgenic and wild-type plants. Data were collected, two days after inoculation with *P. capsici*. (C) Phenotypes of transgenic and wild-type plants after inoculation with *P. capsici* three days. The black arrow indicates the phenotype of the left transgenic and wild-type plants after removing the matrix. White arrows indicate the constricted area between root and stem. (D) Classification of disease index percentage of transgenic and wild-type plants after inoculation with *P. capsici* thirteen days. (E) Disease index percentage of transgenic and wild-type plants and data were collected thirteen days after inoculation with *P. capsici*. (F) The expression level of CaSBP08 in transgenic and wild-type plants. (G) The expression of defense-related genes in transgenic and wild-type plants after inoculation with *P. capsici*. Bars in Figure A are 0.4cm, C and D are 3.5cm. * and ** represent significant differences at $P \leq 0.05$ and $P \leq 0.01$ respectively. Mean values and SDs for three biological replicates are shown.
CaSBP13) that are involved in plant defense responses to *P. capsici* infection. To further study the function of CaSBP genes in the process of *P. capsici* infection response, we chose one of our screened peppers SBP-box gene (CaSBP08) for further research.

CaSBP08 has a 336-bp open reading frame, encoding 111 amino acids (Zhang et al., 2016). The CaSBP08 protein was localized to the nucleus (Figure 3). Silencing of this gene enhanced resistance to *P. capsici* infection in pepper plants.

After *P. capsici* inoculation, the lesion areas of detached leaves of CaSBP08-silenced plants were smaller than the lesion areas of the negative control plants (Figures 4A, B). The disease index percentage of CaSBP08-silenced plants was also lower than that of the negative control plants (Figure 4D). Furthermore, after inoculation with *P. capsici* using the root-drench method, the MDA content as well as the POD and CAT activities of CaSBP08-silenced plants increased but were lower than those of the negative control treatment plants (Figure 4E). A plant under stress is closely related to membrane lipid peroxidation, which is induced by active oxygen accumulation. MDA is one of the most important products of membrane lipid peroxidation. Therefore, the degree of membrane lipid peroxidation can be determined by measuring MDA content. This can reflect the degree of damage to the membrane system and the resistance of plants (Ma et al., 2013). Moreover, it has been reported that during *Phytophthora* root rot development there is a relationship between the disease induced by *P. capsici* and the antioxidant system (Koç and Üstün, 2012). The protein encoded by the peroxidase CanPOD gene plays a positive role in plant defense responses to *P. capsici* infection in pepper plants (Wang et al., 2013b), as CanPOD is related to reactive oxygen species (ROS)-scavenging enzymes. CAT is also a major ROS-scavenging enzyme in plants (Mittler et al., 2004). POD and CAT activity levels were increased during *P. capsici* infection in pepper plants (Koç and Üstün, 2012). The high POD activity indicates that pepper plants are extra sensitive to infection by *P. capsici* as POD-mediated enzymatic reactions are enhanced in infected plants (Wu et al., 2016). POD activity increase is an essential factor in enhancing resistance to plant disease (Wu et al., 2016). The activity of POD increases during the first stage of *Xanthomonas campestris pv. vesicatoria* infection and then declines when the H$_2$O$_2$ accumulation reaches its maximum in pepper plants (Do et al., 2003). After *P. capsici* inoculation, the increased rate of POD activity in susceptible varieties was greater than that in resistant varieties of Kernel Pumpkin (Zhou et al., 2003). Moreover, catalase plays a key role in maintaining H$_2$O$_2$ homeostasis in cells and has been implicated in ROS signaling in response to pathogen attack (Magbanua et al., 2007). In addition, during infection, *Phytophthora nicotianae* increases its own peroxisomal catalase levels while concurrently down-regulating host catalase expression (Blackman and Hardham, 2008). The activity of CAT in cucumber varieties resistant to downy mildew was lower compared with susceptible varieties (Wang, 2001).

It has been reported that the strong suppression of pepper CaPO1 can cause dramatic H$_2$O$_2$ accumulation and a huge decrease in peroxidase activity during programmed cell death (Do et al., 2003). Therefore, we detected the expression level of CaPO1 subsequent to *P. capsici* inoculation into pepper plants. The level of CaPO1 expression was suppressed in the negative control plants and first increased and then decreased in the CaSBP08-silenced plants (Figure 5). Bae et al. (2006) reported that poplar PoPOD1 is suppressed under NaCl, methylviologen, polyethylene glycol, gibberellic acid (GA3) and jasmonic acid (JA) treatments.

Furthermore, it has been reported that SA- and JA-mediated signal transduction pathways play a crucial role in plant resistance to diseases (Spoel et al., 2003). Most CaSBP genes can be induced under SA and MeJA treatment and inhibited during the early stage by SA synthesis inhibitor (p-acetylbutrazol, PBZ) and MeJA (salicylhydroxamic acid, SHAM) synthesis inhibitor treatments (Zhang et al., 2016). To determine whether CaSBP08 is involved in the SA- and MeJA-mediated resistant pathways, we also studied and detected the expression of some defense-related genes. For example, the molecular marker CaSAR8.2 can be used for the detection of several pathogenic diseases that affect the SA-mediated signal transduction pathway (Lee and Hwang, 2003). CaBPR1 is involved in the hypersensitive response and was induced in an incompatible interaction of leaves with *Xanthomonas campestris pv. vesicatoria* (Kim and Hwang, 2000). CaDEF1 is involved in the MeJA-mediated signal transduction pathway, which has functions in microbial infection, as abiotic elicitors, and in response to some environmental stressors (Do et al., 2004). In this work, the CaSAR8.2 and CaBPR1 expression levels were induced at the first stage and then decreased in CaSBP08-silenced plants, while they were suppressed in the negative control plants (Figure 5). Furthermore, the expression level of CaDEF1 increased in negative control plants and CaSBP08-silenced plants and was higher in CaSBP08-silenced plants compared with negative control plants. The results demonstrate the involvement of CaSBP08 in the SA- and MeJA-mediated resistant pathways. Further research, however, is need in this regard.

Ectopic expression of *BpSPL9* in the *Betula platyphylla* Suk. (birch) has been reported to enhance the ROS scavenging under drought and salt stress (Ning et al., 2017). *SPL9* interacts with JA ZIM-domain (IAZ) proteins and negatively regulates JA response, though it promotes JAZ3 accumulation in *Arabidopsis* (Mao et al., 2017). Similarly, *VpsSBP5* likely participates in regulating resistance against *Erysiphe necator* though SA- and MeJA-mediated signal transduction pathway in grapes (Hou et al., 2013). Overexpression of CaSBP08 in transgenic *N. benthamiana* enhanced susceptibility to *P. capsici* infection, as demonstrated by the higher average disease area and disease index percentage compared to WT plants (Figures 6B, E). Besides, the *NhPR1a* and *NhPR1b* genes, which are involved in the SA-induced systemic acquired resistance pathway and JA-mediated disease resistance signaling pathway, respectively, were induced in the CaSBP08 transgenic and WT plants (Sohn et al., 2007; Cheol Song et al., 2016). *PR1a* and *PR1b*, two reported defense-related genes in tobacco, can be highly induced by tobacco mosaic virus (TMV) (van Huijsduijnen et al., 1985). The expression level of the SA signaling marker gene *NhPR1a* was associated with systemic acquired resistance (SAR) against...
Pseudomonas syringae pv. tabaci (Cheol Song et al., 2016). PR1b is a JA-responsive gene in tobacco (Sohn et al., 2007). Moreover, it has been reported that ectopic overexpression of CaS3H14 enhances resistance of tobacco toRalstonia solanacearum infection, and the expression of PR1b was induced in transgenic and WT plants. However, the expression of PR1b in transgenic lines was significantly lower than that in WT plants (Qiu et al., 2018). The results of the present study suggest that CaSBP08 may be involved in resistance to P. capsici through regulating the expression of defense-related genes. However, further research is need to examine the regulatory mechanism. In N. benthamiana, SPL6 plays a positive regulatory role in nucleotide binding rich leucine repeat (N TIR-NB-LRR) receptor-mediated plant natural immunity (Padmanabhan et al., 2013). Overexpression of JcNAC1 in Jatropha curcas can enhance susceptibility of plants to Botrytis cinerea infection and inhibit the expression of some defense-related marker genes (Qin et al., 2014). Overexpression of ATAF2 in Arabidopsis can enhance the sensitivity of plants to Fusarium oxysporum infection and repress the expression of pathogenesis-related genes (Delessert et al., 2005).

CONCLUSIONS

In conclusion, we screened four genes (CaSBP08, CaSBP11, CaSBP12, and CaSBP13) out of the fifteen identified CaSBP genes, each of which responded to P. capsici infection. Additionally, we selected one of our screened pepper SBP-box genes (CaSBP08) for further research. CaSBP08 protein was thus observed to be localized to the nucleus. Silencing CaSBP08 enhanced resistance against P. capsici infection, such that the average disease area, the percent disease index, and the POD and CAT activities were lower in the CaSBP08-silenced plants compared with the negative control plants. Additionally, following inoculation with P. capsici, the defense genes CaPO1, CaDEF1, CaBPR1, and CaSAR8.2 were induced during the early stage of infection in CaSBP08-silenced plants, while CaPO1, CaBPR1, and CaSAR8.2 were suppressed in the negative control plants. In addition, overexpression of CaSBP08 in N. benthamiana enhanced susceptibility to P. capsici infection, as demonstrated by the average disease area and the percent disease index being greater than those of WT plants. Our work provides a basis for future research on the role of CaSBP genes in plant resistance to infections by P. capsici and similar pathogens.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

H-XZ and Z-HG perceived and designed research. H-XZ, X-HF, and J-HJ carried out the experiments. H-XZ analyzed data. H-XZ wrote the manuscript. MA and AK revised the manuscript. Z-HG contributed reagents/materials/analysis tools. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00183/full#supplementary-material

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