The pepper Bs4C proteins are localized to the endoplasmic reticulum (ER) membrane and confer disease resistance to bacterial blight in transgenic rice

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SUMMARY

Transcription activator-like effector (TALE)-dependent dominant disease resistance (R) genes in plants, also referred to as executor R genes, are induced on infection by phytopathogenic bacteria of the genus Xanthomonas harbouring the corresponding TALE genes. Unlike the traditional R proteins, the executor R proteins do not determine the resistance specificity and may function broadly in different plant species. The executor R gene Bs4C-R in the resistant genotype PI 235047 of the pepper species Capsicum pubescens (CpBs4C-R) confers disease resistance to Xanthomonas campestris pv. vesicatoria (Xcv) harbouring the TALE genes avrBsP/avrBs4. In this study, the synthetic genes of CpBs4C-R and two other Bs4C-like genes, the susceptible allele in the genotype P158270 of C. pubescens (CpBs4C-S) and the CaBs4C-R homologue gene in the cultivar ‘CM334’ of Capsicum annuum (CaBs4C), were characterized in tobacco (Nicotiana benthamiana) and rice (Oryza sativa). The Bs4C genes induced cell death in N. benthamiana. The functional Bs4C-eCFP fusion proteins were localized to the endoplasmic reticulum (ER) membrane in the leaf epidermal cells of N. benthamiana. The Xa10 promoter-Bs4C fusion genes in transgenic rice conferred strain-specific disease resistance to Xanthomonas oryzae pv. oryzae (Xoo), the causal agent of bacterial blight in rice, and were specifically induced by the Xa10-incompatible Xoo strain PX0996 (pHM1avrXa10). The results indicate that the Bs4C proteins from pepper species function broadly in rice and the Bs4C protein-mediated cell death from the ER is conserved between dicotyledonous and monocotyledonous plants, which can be utilized to engineer novel and enhanced disease resistance in heterologous plants.

Keywords: bacterial blight, Bs4C, R gene, rice, TAL effector, Xa10.

INTRODUCTION

Transcription activator-like effectors (TALEs) comprise a large family of bacterial type III effectors with sequence-specific DNA binding activity, found in many species and pathovars of the genus Xanthomonas, with more distant orthologues inRalstonia solanacearum and Burkholderia rhizoxinica, where they play important roles in host–pathogen interactions (Boch and Bonas, 2010; Juillerat et al., 2014; Lange et al., 2013, 2014). TALEs share a highly conserved tripartite protein structure (Boch and Bonas, 2010; Schreiber et al., 2015). The N-terminal region of TALEs harbours signals for secretion through the type III secretion system and translocation into the plant cell. The C-terminal region contains a host transcription factor binding domain (Yuan et al., 2016), nuclear localization signals and an acidic activation domain. TALEs differ mostly in the central DNA binding domain, which is composed of nearly identical tandem repeats of typically 34 amino acids with repeat-variable di-residues (RVDs) at positions 12 and 13 that determine the DNA binding specificity (Boch et al., 2009; Deng et al., 2012; Mak et al., 2012; Moscou and Bogdanove, 2009). TALEs bind specifically to short DNA elements, also termed effector binding elements (EBEs), in the promoters of the targeted host genes in a ‘one RVD to one nucleotide’ manner, and activate gene expression (Boch et al., 2009; Moscou and Bogdanove, 2009). The bacteria usually use TALEs to target host susceptibility (S) genes for disease development (Antony et al., 2010; Cernadas et al., 2014; Hu et al., 2014; Kay et al., 2007; Streubel et al., 2013; Sugio et al., 2007; Yang et al., 2006; Yu et al., 2011; Zhou et al., 2015). However, plants have co-evolved disease resistance (R) genes to counteract bacterial infection by taking advantage of TALE activity. Three types of TALE-dependent R genes have been reported, which confer recessive, dominant non-transcriptional- and dominant transcriptional-based resistance. TALE-dependent recessive resistance occurs in plants with DNA polymorphisms of EBEs in S gene promoters, which enable the S genes to avoid being targeted by TALEs (Chu et al., 2006; Zhou et al., 2015). The dominant non-transcriptional-based resistance is represented solely by the classical nucleotide binding site-leucine-rich repeat (NBS-LRR) resistance gene from tomato, Bs4, which was identified as the cognate R gene to the TALE genes.

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avrBsPavrBs4 (Bonas et al., 1993; Schornack et al., 2004). The transcrip-
tional activity of the TALEs is not required for Bs4 resistance elicitation as the truncated versions of AvrBs4 also trigger disease resistance (Schornack et al., 2004). The dominant transcrip-
tional-based R genes, also referred to as executor R genes for cell death (Bogdanove et al., 2010), are directly targeted and activated by TALEs, and the expressed R proteins trigger a hypersensitive response (HR) for disease resistance (Gu et al., 2005; Römer et al., 2007; Strauss et al., 2012; Tian et al., 2014; Wang et al., 2015). It should be noted that the specificities of the executor R gene-mediated disease resistance are determined by the EBEs in the promoters of R genes, rather than by the R protein.

Bacterial spot of pepper and tomato, caused by Xanthomonas campestris pv. vesicatoria (Xcv) (Thieme et al., 2005), and bacterial blight of rice, caused by Xanthomonas oryzae pv. oryzae (Xoo) (Salzberg et al., 2008), are two devastating diseases that severely affect the commercial production of these crops. In both pathosys-
tems, TALEs from Xcv or Xoo strains contribute to virulence by targeting to host S genes or interfere with the host resistance response (Antony et al., 2010; Ji et al., 2016; Kay et al., 2007; Streubel et al., 2013; Sugio et al., 2007; Yang et al., 2006; Yu et al., 2011; Zhou et al., 2015), whereas plants have co-evolved different types of R genes to counteract bacterial infection (Chu et al., 2006; Gu et al., 2005; Iyer and McCouch, 2004; Römer et al., 2007; Schornack et al., 2004; Strauss et al., 2012; Tian et al., 2014; Wang et al., 2015). Three executor R genes, Xa10, Xa23 and Xa27, have been isolated from rice, and their expression confers race-specific disease resistance to Xoo strains harbou-
ring the cognate TALE genes avrXa10, avrXa23 and avrXa27 (Gu et al., 2005; Tian et al., 2014; Wang et al., 2015). The Xa27 gene encodes a compact executor R protein with unknown biochemical function, which is localized to the apoplast (Gu et al., 2005; Wu et al., 2008). The gene products of Xa10 and Xa23 form a small executor R protein family (Tian et al., 2014; Wang et al., 2015). They locate to the endoplasmic reticulum (ER) membrane by forming oligomers, where they trigger cell death by disrupting the ER and cellular Ca$^{2+}$ homeostasis (Tian et al., 2014; Wang et al., 2017). Unlike Xa27, which only triggers HR in rice, the Xa10/Xa23 family proteins induce cell death in both rice and Nicotiana benthamiana (Tian et al., 2014; Wang et al., 2017). Two executor R genes, Bs3 and Bs4C-R, have been isolated from pepper (Römer et al., 2007; Strauss et al., 2012). The Bs3 gene in the species Capsicum annuum encodes a flavin-dependent monoxygenase (Römer et al., 2007). The Bs4C-R gene in the genotype PI 235047 of the species Capsicum pubescens (CpBs4C-R) encodes a puta-
tive 164-amino-acid protein that shares no significant homology to any other protein of known function (Strauss et al., 2012). The Bs4C-R gene in pepper mediates the recognition of the TALE protein AvrBs4 from Xcv (Strauss et al., 2012). As mentioned above, AvrBs4, acting as an avirulence gene product, also triggers Bs4 dependent disease resistance to Xcv in tomato (Schornack et al., 2004). Genomes of solanaceous species contain Bs4C-like genes, such as the susceptible allele in the genotype PI 585270 (CpBs4C-S) and the Bs4C-R homologue gene in the C. annuum cultivar ‘CM334’ (CaBs4C), and these were found to be under tight transcrip-
tional control (Strauss et al., 2012). As part of an effort to characterize the functions of the Bs4C genes, we report the results on the subcellular localization of the three Bs4C proteins (CaBS4C, CpBs4C-R and CpBs4C-S) in N. benthamiana and their function in heterologous and monocotyledonous rice plants for disease resistance to bacterial blight.

**RESULTS**

The synthetic Bs4C genes induce cell death in N. benthamiana

The coding regions of the three Bs4C genes (CaBS4C, CpBs4C-R and CpBs4C-S) were chemically synthesized based on their genomic sequence published previously (Strauss et al., 2012) (Sequences S1–S3). The codons of the synthetic Bs4C genes were optimized to facilitate their expression in rice. The deduced gene products of the synthetic Bs4C genes are identical to those encoded by the native Bs4C genes in C. annuum (CaBs4C) or C. pubescens (CpBs4C-R and CpBs4C-S). To avoid redundancy, the synthetic Bs4C genes were designated as CaBs4C, CpBs4C-R and CpBs4C-S, respectively. The gene products of the three Bs4C genes (CaBS4C, CpBs4C-R and CpBs4C-S) share high identity at the amino acid level (Fig. 1a). The Bs4C genes were fused with the CaMV 35S promoter (P35S) and nopaline synthase gene termi-
nator (Tnos) to generate the fusion genes P35S:CaBS4C-Tnos, P35S:CpBs4C-R-Tnos and P35S:CpBs4C-S-Tnos. The Bs4C fusion genes were transiently expressed in N. benthamiana by agroinfiltration. At 24 h after infiltration (HAI), cell death was observed in N. benthamiana infiltrated with agrobacteria harbouring binary constructs containing P35S:CaBs4C-Tnos, P35S:CpBs4C-R-Tnos or P35S:CpBs4C-S-Tnos (Fig. 2). No cell death was observed in N. benthamiana infiltrated with agrobacteria harbouring the empty vector (Fig. 2). The Bs4C gene-induced cell death in N. benthamiana was further confirmed by trypan blue staining (Fig. 2). Previous reports have demonstrated that the constitutive or inducible expression of the Bs4C-R or Bs4C-S gene (CpBs4C-R or CpBs4C-S in this study) in N. benthamiana triggers cell death (Strauss et al., 2012). The results in this study indicate that, like CpBs4C-R or CpBs4C-S, CaBs4C encodes a functional executor R protein that also triggers cell death in N. benthamiana.
The Bs4C proteins are localized to the ER membrane

Protein structure prediction indicated that the Bs4C proteins were putative transmembrane proteins with each containing four predicted transmembrane helices and showing similar topography on membranes to that of the Xa10 protein, even though members of the two executor R protein families shared no identity at the amino acid level (Fig. 1b) (Tian et al., 2014; Wang et al., 2017). To detect the subcellular localization of the three Bs4C proteins, the Bs4C genes were fused in-frame with the enhanced cyan fluorescent protein gene (eCFP). The 35S-driven Bs4C fusion genes (P35S:CaBs4C-eCFP:Tnos, P35S:CpBs4C-R-eCFP:Tnos and P35S:CpBs4C-S-eCFP:Tnos) induced cell death in N. benthamiana (data not shown), indicating that the Bs4C-eCFP fusion proteins (CaBs4C-eCFP, CpBs4C-R-eCFP and CpBs4C-S-eCFP) were functional. The Bs4C fusion genes were then co-expressed with the ER membrane marker gene P35S:eYFP-RcDGAT2:Tnos in N. benthamiana through agroinfiltration (Tian et al., 2014). A confocal microscopy study demonstrated that the Bs4C-eCFP fusion proteins and eYFP-RcDGAT2 were co-localized to the ER membrane in leaf epidermal cells of N. benthamiana.
Agrobacterium produced transgenic rice in the cultivar Nipponbare background via constitutive or leaky expression of indicated that these stress-related phenotypes resulted from retardation of growth and development. Molecular analysis should be mentioned that most of the resistant transgenic plants shown). The expression of the executor genes in rice, such as Xa10 and Xa27, is tightly controlled in the absence of the corresponding TALEs, but their transgenes in transgenic plants were frequently found to show constitutive or leaky expression, possibly because of the position effect of the transgenes in the rice genome (Gu et al., 2005; Zeng et al., 2015). Transgenic line 50 of PXa10:CaBs4C:TXa10 (L50), line 64 of PXa10:CpBs4C-R:TXa10 (L64) and line 120 of PXa10:CpBs4C-S:TXa10 (L120) displayed a normal morphological phenotype in growth and development and conferred disease resistance to PXO99A(pHM1avrXa10). They were selected for further molecular and genetic studies. The T3 plant of L50 contained at least eight copies of T-DNA (data not shown). A T3 plant of L50 (T0-50/T1-31/T2-25/T3-23) was identified to contain three to four copies of T-DNA detected by the Hpt probe (Figs 5 and S1, see Supporting Information). At least one copy of the T-DNAs contained the intact PXa10:CaBs4C:TXa10 gene by producing the expected 4.7-kb PstI-XbaI band, detected by the PXa10 probe in Southern blot analysis (Fig. 5). The other copies of the T-DNAs in L50/T3-23 produced three bands with a molecular size larger than 4.7 kb (Fig. 5), which might result from DNA mutation or rearrangement during T-DNA integration. Using a similar approach, a T1 plant of L64 (L64/T1-27) and a T1 plant of L120 (L120/T1-95) were identified to contain intact PXa10:CpBs4C-R:TXa10 and PXa10:CpBs4C-S:TXa10 genes, respectively (Figs 5 and S1). T0-64/T1-27 carried two copies of T-DNAs, and one copy of the T-DNAs contained the intact PXa10:CpBs4C-R:TXa10 gene (Fig. 5). L120/T1-95 carried one copy of T-DNA that contained the intact PXa10:CpBs4C-S:TXa10 gene (Fig. 5).

Transgenic Bs4C rice plants confer AvrXa10-dependent disease resistance to Xoo strains

The progeny of L50/T3-23, L64/T1-27 and L120/T1-95 that contained the intact 4.7-kb B4S4 fusion genes were inoculated with PXO99A(pHM1) and PXO99A(pHM1avrXa10). Transgenic Bs4C plants (T4 plants of L50 of PXa10:CaBs4C:TXa10 or L50/T4, T2 plants of L64 of PXa10:CpBs4C-R:TXa10 or L64/T2, and T2 plants of L120 of PXa10:CpBs4C-S:TXa10 or L120/T2) conferred specific resistance to PXO99A(pHM1avrXa10), but were susceptible or moderately susceptible to PXO99A(pHM1) (Fig. 6 and Table 2). In a control experiment, Nipponbare was susceptible to both PXO99A(pHM1)
and PXO99A(pHM1avrXa10) (Fig. 6 and Table 2). The results indicate that the Bs4C genes under the control of the Xa10 promoter in transgenic Bs4C rice plants confer AvrXa10-dependent resistance to PXO99A(pHM1avrXa10).

The AvrXa10-dependent Bs4C induction in transgenic rice plants was detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The expression of \( P_{\text{Xa10}}: \text{CaBs4C}:T_{\text{Xa10}} \) was almost undetectable in non-inoculated L50/T4 plants, whereas very low levels of gene expression of \( P_{\text{Xa10}}:\text{CpBs4C-R}:T_{\text{Xa10}} \) were detected in non-inoculated L64/T2 plants and of \( P_{\text{Xa10}}:\text{CpBs4C-S}:T_{\text{Xa10}} \) in non-inoculated L120/T2 plants (Fig. 7). The expression of transgenic Bs4C genes was

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*Fig. 3* Subcellular localization of the Bs4C proteins in leaf cells of *Nicotiana benthamiana*. The endoplasmic reticulum (ER) membrane marker eYFP-RcDGAT2 was transiently co-expressed with eCFP, CaBs4C-eCFP, CpBs4C-R-eCFP or CpBs4C-S-eCFP in leaf epidermal cells of *N. benthamiana*. Images were taken at 24 h after infiltration. Bars, 10 \( \mu \)m. eCFP, enhanced cyan fluorescent protein; eYFP, enhanced yellow fluorescent protein.
in the L50/T4, L64/T2 and L120/T2 plants at 48 h after inoculation with PXO99A(pHM1avrXa10) (Fig. 7). No gene induction of Bs4C transgenes was detected in the three transgenic lines at 48 h after inoculation with strain PXO99A(pHM1), and their expression levels were similar to those of non-inoculated transgenic plants (Fig. 7). The results indicate that the Bs4C transgenes in rice are specifically induced in the presence of AvrXa10 from PXO99A(pHM1), and their expression levels were similar to those of non-inoculated transgenic plants (Fig. 7). The results also demonstrate that the disease resistance genes CaBs4C, CpBs4C-R and CpBs4C-S from the dicot plant pepper function broadly in the monocot plant rice.

**DISCUSSION**

Except for Xa10 and Xa23, other executor R proteins show great diversity at the amino acid level (Gu et al., 2005; Römer et al., 2007; Strauss et al., 2012; Tian et al., 2014; Wang et al., 2015, 2017). With regard to subcellular localization, Bs3 is a flavin-dependent monooxygenase and the GFP-tagged Bs3 (Bs3-GFP) protein was observed in both the cytoplasm and nuclei of leaf cells of N. benthamiana (Römer et al., 2007). Xa27 was found to be translocated to the apoplast of the plant cells and a signal-anchor-like sequence at the N-terminal region of Xa27 was required for its translocation and disease resistance to Xoo (Wu et al., 2008). Xa10 proteins are localized to the ER membrane by forming hexamers or higher oligomers, and this may also be true for Xa23 proteins (Tian et al., 2014; Wang et al., 2017). In this study, we found that the eCFP-tagged Bs4C proteins, CaBs4C-eCFP, CpBs4C-R-eCFP and CpBs4C-S-eCFP, were localized to the ER membrane (Fig. 3). The ER is an essential organelle of eukaryotic cells that is involved in multiple cellular processes, including calcium homeostasis, protein secretion and lipid biosynthesis. A few ER-resident proteins have been identified as cell death regulators involved in plant innate immunity (Caplan et al., 2009; Carvalho et al., 2014; Liebrand et al., 2012; Tian et al., 2014; Xu et al., 2012; Zhu et al., 2010). Our previous studies have demonstrated that the overexpression of ER membrane-localized Xa10 or Xa23 in N. benthamiana induces the depletion of Ca2+ from the ER lumen (Tian et al., 2014; Wang et al., 2017). However, no such activity was detected with the three Bs4C proteins in this study (data not shown).

Although Bs4C and Xa10 proteins might function via different cell death signalling from the ER, the ER-localized executor R

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**Table 1** Number of transgenic T0 plants obtained from rice transformation with Bs4C genes.

| Gene                        | Total | Resistant | Susceptible |
|-----------------------------|-------|-----------|-------------|
| PXa10:CaBs4C:TXa10          | 25    | 18        | 7           |
| PXa10:CpBs4C-R:TXa10        | 115   | 83        | 32          |
| PXa10:CpBs4C-S:TXa10        | 103   | 82        | 21          |

*Six-week-old transgenic T0 plants were inoculated with PXO99A(pHM1avrXa10) using the leaf-clipping method and bacterial blight lesions were measured at 14 days after inoculation.
late blight, by the inducible cell death controlled by barnase and rice (Zhao et al., 2005) as the causal agent of bacterial leaf streak of rice, when introduced into the fungus. A successful example is the inhibition of fungal sporulation through genetic engineering to mimic the HR of plant cells for disease resistance. Resistance against Xanthomonas oryzae pv. oryzae (Xoo) strains PXO99A(pHM1) and PXO99A(pHM1avrXa10). The inoculated leaves were photographed at 14 days after inoculation. Ni, Nipponbare; L50/T4, T2 plant of transgenic line 50 of Pka10-CaBs4C-Txa10; L64/T2, T2 plant of transgenic line 64 of Pka10-CpBs4C-R-Txa10; L120/T2, T2 plant of transgenic line 120 of Pka10-CpBs4C-S-Txa10.

Table 2 Lesion length and disease phenotype of transgenic rice plants at 14 days after inoculation with PXO99A(pHM1) and PXO99A(pHM1avrXa10).

| Plant        | Gene                          | Lesion length (cm) | Disease score* |
|--------------|-------------------------------|--------------------|----------------|
| Nipponbare   | N.A.                          | 11.6 ± 2.1 (S)     | 12.1 ± 2.0 (S) |
| L50          | Pka10-CaBs4C-Txa10            | 13.1 ± 4.2 (S)     | 1.4 ± 1.1 (R)  |
| L64          | Pka10-CpBs4C-R-Txa10          | 8.7 ± 2.5 (MS)     | 0.7 ± 0.4 (R)  |
| L120         | Pka10-CpBs4C-S-Txa10          | 6.4 ± 2.5 (MS)     | 0.6 ± 0.3 (R)  |

*Six-week-old plants were inoculated with Xanthomonas oryzae pv. oryzae (Xoo) strains using the leaf-clipping method and bacterial blight lesions were measured at 14 days after inoculation. The lesion length of bacterial blight is the average of 16 infected leaves from four inoculated plants. The standard deviation of the mean is indicated. MS, moderately susceptible; N.A., N.A.; R, resistant; S, susceptible.

proteins function broadly across monocotyledonous and dicotyledonous plants, which made it possible for us to engineer AvrXa10/EBEavrXa10-dependent and Bs4C-mediated transgenic resistance in rice. Transgenic resistance has long been used as a method for the control of plant diseases (Cillo and Palukaitis, 2014; Saharan et al., 2016). Successful genetic engineering by the introduction of R genes from unrelated plant species has been reported to generate broad-spectrum resistant plants (Tai et al., 1999; Thilmony et al., 1995; Witham et al., 1996; Zhao et al., 2005).

For example, the R gene Rxo1 from maize has been demonstrated to confer resistance against Xanthomonas oryzae pv. oryzae (Xoo), the causal agent of bacterial leaf streak of rice, when introduced into rice (Zhao et al., 2005). The generation of controlled cell death through genetic engineering to mimic the HR of plant cells for disease resistance is another approach to generate transgenic resistance. A successful example is the inhibition of fungal sporulation of the fungus Phytophthora infestans, the causal agent of potato late blight, by the inducible cell death controlled by barnase and bastar in transgenic potato plants (Strittmatter et al., 1995). The native function of TALE-dependent R gene products as executors of HR or programmed cell death in plants makes them ideal candidates for the engineering of controlled cell death in distantly related plant species. In this study, transgenic rice plants carrying Bs4C genes conferred specific disease resistance to PXO99A(pHM1avrXa10), and Bs4C transgenes in transgenic plants were specifically induced by AvrXa10 from the incompatible pathogen. For example, the interaction between AvrXa10 and EBEavrXa10 in the Xa10 promoter determined the recognition specificity, whereas the induced Bs4C proteins triggered HR and disease resistance.

This proof-of-concept study, together with previous studies (Tian et al., 2014; Wang et al., 2015, 2017), indicates that executor R proteins, such as Bs4C and Xa10, can be used to engineer transgenic resistance in distantly related plant species against phytopathogenic bacteria that rely on TALE or TALE-like effectors for virulence. By changing and adding multiple EBEs in the promoters of the executor R genes, novel and broad-spectrum resistance specificity could be generated in desirable plant species (Hummel et al., 2012; Zeng et al., 2015).

**EXPERIMENTAL PROCEDURES**

**Rice line and growth condition**

Nipponbare is a cultivar of japonica rice, which is susceptible to many Xoo strains. Rice plants were grown in a glasshouse at a temperature of 30 °C for 12.5 h (light) and 26 °C for 11.5 h (dark) with average humidity at 84%.

**Genes and constructs**

The DNA sequences of the coding regions of the CaBs4C, CpBs4C-R and CpBs4C-S genes were synthesized by GenScript (Piscataway, NJ, USA) after codon optimization for gene expression in rice (Sequences S1–S3). The coding regions of the synthetic CaBs4C, CpBs4C-R and CpBs4C-S
genes were fused with the Xa10 promoter through PCR amplification. The 1196-bp BamHI-Apal fragment from rice genomic DNA was digested with appropriate restriction enzymes. The digested DNA samples were separated completely on 0.8% agarose gel and blotted to Hybond™-N+ nylon membrane (Amersham Biosciences, Piscataway, NJ, USA). DNA hybridization and detection of the interested genes or DNA fragments were conducted using the DIG DNA labelling and detection kit (Roche Applied Science, Penzburg, Upper Bavaria, Germany) in accordance with the manufacturer’s instructions. The DNA probe for detecting the copy number of T-DNA was the hpt probe derived from the coding region of the hpt gene, and the common DNA probe for detecting the fusion Bs4C genes was the P20 probe derived from the Xa10 promoter (Fig. 4). The oligo DNA primer pairs were Hpt-F(5’-AAAAGCCTGAACCTCAGCGG-3’) and Hpt-R(5’-GGTTGAGAGC-3’); L50/T4, T4 plant of transgenic line 50 of P20; L120/T2, T2 plant of transgenic line 64 of P20, respectively. qRT-PCR experiments were performed in triplicate, and the data are presented as the means ± standard deviation (SD). L50/T4, T4 plant of transgenic line 50 of P20; L120/T2, T2 plant of transgenic line 64 of P20.

qRT-PCR

Total RNA was extracted from rice leaf tissues using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). About 1 µg of total RNA was treated by DNase I. The first-strand cDNAs were synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. qPCR was conducted using a CFX96 real-time PCR system (Bio-Rad). A standard reaction mixture (20 µL) contained 1 µL cDNA template, 10 µL KAPA SYBR® FAST qPCR Master Mix (2X) Universal (KAPA-BIOSYSTEMS, Boston, MA, USA) and 200 nM forward and reverse primers. The PCR was conducted with an initial denaturing step of 95 °C for 3 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. PCR product specificity was confirmed by melting curve analysis and agarose gel electrophoresis to ensure that the PCRs were free of primer dimers. The expression of the rice ubiquitin gene 5 (OsUbi5) was used as the internal control. The qRT-PCR experiments were conducted in triplicate and the data are presented as the means ± standard deviation (SD). The oligo DNA primers were CaBs4C-Q-F(5’-ACACCCCAATCAGGAACG-3’); CaBs4C-Q-R(5’-GCCATCGGT-3’); Os50(5’-GGTTGAGAGC-3’). The expression of the rice ubiquitin gene 5 (OsUbi5) was used as the internal control. The qRT-PCR experiments were conducted in triplicate and the data are presented as the means ± standard deviation (SD). The oligo DNA primers were CaBs4C-Q-F(5’-ACACCCCAATCAGGAACG-3’); CaBs4C-Q-R(5’-GCCATCGGT-3’); Os50(5’-GGTTGAGAGC-3’).
CG3)\textsuperscript{a}/CaBs4C-Q-R:5'TATCCGTACCACTGACAGAGA3'\textsuperscript{b} for the \textit{PXa10-CaBs4C-T\textsubscript{Xa10}} gene, \textit{CpBs4C-Q-R:5'(TAGCGCTCTGGCCGATCGTCTT3')/CpBs4C-TXa10} for the \textit{PXa10-CpBs4C-R:TXa10} gene, \textit{CpBs4C-S-Q-F:5'(CATCAGGATCGCTCAGTT3')/CpBs4C-S:TXa10} gene and \textit{UBQ5-F:5'(AACACCTGGACGCCCACT3')/UBQ5-R:5'GTGCGATTTCCCTTCC TTC3'} for the \textit{Oslb1b5} gene.

**Agroinfiltration of \textit{N. benthamiana} and confocal microscopy**

\textit{Agrobacterium tumefaciens} strain GV3101 harbouring binary constructs was cultured in 5 mL of LB liquid medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, pH 7.0) with appropriate antibiotics at 28 °C until the bacteria reached an optical density at 600 nm (\textit{OD}\textsubscript{600}) of 0.8, and then subcultured in 100 mL of AB liquid medium (3 g/L K\textsubscript{2}HPO\textsubscript{4}, 1 g/L NaH\textsubscript{2}PO\textsubscript{4}, 1 g/L NH\textsubscript{4}Cl, 0.15 g/L KCl, 0.01 g/L CaCl\textsubscript{2}, 0.0025 g/L FeSO\textsubscript{4}7H\textsubscript{2}O, 5 g/L glucose, pH 7.0) at 28 °C until the bacteria reached \textit{OD}\textsubscript{600} = 0.8. The bacteria were collected and resuspended in 11 mM MgCl\textsubscript{2} to \textit{OD}\textsubscript{600} = 0.6–0.8. 2-(\textit{N}-Morpholino)ethanesulfonic acid (MES) and acetylsyringone (AS) were then added to the bacterial solution at final concentrations of 10 mM and 200 \textmu M, respectively. \textit{Nicotiana benthamiana} plants were grown in a growth room with 16 h of light and 8 h of dark at 25 °C. Leaves of 4-week-old \textit{N. benthamiana} plants were used for infiltration as described previously (Kay \textit{et al.}, 2007). For confocal microscopy, leaf samples were examined on an LSM 510 Exciter Upright confocal microscope (Carl Zeiss, Oberkochen, Germany). The excitation/emission combinations were 405/475–525-nm band pass for eCFP, 514/610-nm band pass for autofluorescence of chlorophyll.

**Trypan blue staining**

The trypan blue staining solution was prepared by diluting trypan blue stock solution (10 g phenol, 10 mL glycerol, 10 mL lactic acid, 10 mL water and 0.02 g trypan) (Sigma-Aldrich, St. Louis, MO, USA) with 96% ethanol (1:2, v/v). Leaves of \textit{N. benthamiana} were boiled in trypan blue staining solution for 1 min and left in the solution at room temperature for 24 h. Stained leaves were subsequently de-stained in chloridrate solution (2.5 g of chloridrate dissolved in 1 mL of distilled water) for 72 h.

**Bacterial blight inoculation**

Xoo strains were cultured on a PSA plate (10 g/L peptone, 10 g/L sucrose, 1 g/L glutamic acid, 16 g/L bacto-agar, pH 7.0) at 28 °C for 48 h. Bacterial cells were collected and resuspended in sterile distilled water to \textit{OD}\textsubscript{600} = 0.5. Bacterial blight inoculation on fully expanded leaves of 6-week-old rice plants was carried out using the leaf-clipping method (Kauffman, 1973). The lesion length was measured 14 days after inoculation. The disease symptoms were scored as resistant (R, lesion length ≤ 3.0 cm), moderately resistant (MR, 3.0 cm < lesion length ≤ 6.0 cm), moderately susceptible (MS, 6.0 cm < lesion length ≤ 9.0 cm) and susceptible (S, lesion length > 9.0 cm). To detect the specific induction of transgenes on inoculation with compatible or incompatible \textit{Xoo} strains, leaves of 4-week-old transgenic plants were infiltrated with bacterial inoculum using a needleless syringe. Leaf tissues were collected at 48 h after infiltration.

**Protein analysis**

The amino acid sequences of Bs4C proteins were aligned using the program CLUSTAL W (https://embnet.vital-it.ch/software/CLUSTALW.html) and the output was shaded using GENEDOC software (http://gedenoc.iformer.com/2.7f). The prediction of the transmembrane helix of Bs4C proteins was performed with the SOSUI program (http://sosui.nagahama-i-bio.ac.jp/sosui).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Table S1 Constructs used in this study.

Fig. S1 Morphology of transgenic rice plants carrying different Bs4C genes. Images were taken at 2 months after sowing. Ni, Nipponbare; L50/T3-23, T3 plant of transgenic line 50 of P_{xa10}:CaBs4C:T_{xa10}; L64/T1-27, T1 plant of transgenic line 64 of P_{xa10}:CpBs4C-R:T_{xa10}; L120/T1-95, T1 plant of transgenic line 120 of P_{xa10}:CpBs4C-S:T_{xa10}.

Sequence S1 Nucleotide sequence of the synthetic Capsicum annuum Bs4C gene (CaBs4C) and the deduced amino acid sequence of CaBs4C.

Sequence S2 Nucleotide sequence of the synthetic Capsicum pubescens Bs4C-R gene (CpBs4C-R) and the deduced amino acid sequence of CpBs4C-R.

Sequence S3 Nucleotide sequence of the synthetic Capsicum pubescens Bs4C-S gene (CpBs4C-S) and the deduced amino acid sequence of CpBs4C-S.