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ORIGINAL PAPER

Positional cloning of *ds1*, the target leaf spot resistance gene against *Bipolaris sorghicola* in sorghum

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Abstract Target leaf spot is one of the major sorghum diseases in southern Japan and caused by a necrotrophic fungus, *Bipolaris sorghicola*. Sorghum resistance to target leaf spot is controlled by a single recessive gene (*ds1*). A high-density genetic map of the *ds1* locus was constructed with simple sequence repeat markers using progeny from crosses between a sensitive variety, bmr-6, and a resistant one, SIL-05, which allowed the *ds1* gene to be genetically located within a 26-kb region on the short arm of sorghum chromosome 5. The sorghum genome annotation database for BTx623, for which the whole genome sequence was recently published, indicated a candidate gene from the Leucine-Rich Repeat Receptor Kinase family in this region. The candidate protein kinase gene was expressed in susceptible plants but was not expressed or was severely reduced in resistant plants. The expression patterns of *ds1* gene and the phenotype of target leaf spot resistance were clearly correlated. Genomic sequences of this region in parental varieties showed a deletion in the promoter region of SIL-05 that could cause reduction of gene expression. We also found two *ds1* alleles for resistant phenotypes with a stop codon in the coding region. The results shown here strongly suggest that the loss of function or suppression of the ds1 protein kinase gene leads to resistance to target leaf spot in sorghum.

Keywords Leucine-Rich Repeat Receptor Kinase (LRR-RK) · Map-based cloning · Pathogen related gene · R-gene · S-gene · Toll-like receptor

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Introduction

Sorghum is a C4 grass that is especially important as forage and as a human staple worldwide, especially in the semiarid tropics because of its tolerance of hot and dry environments. Sorghum has recently been promoted for biomass production due to its rapid growth in summer in temperate zones.

Target leaf spot is a disease of sorghum caused by the fungus *Bipolaris sorghicola*. Target leaf spot causes a significant decrease in yield where plants are located in high humidity conditions during the growing season. This disease is prevalent and occasionally severe in the USA (Dalmacio 2000), and it is one of the most devastating diseases in southern

Japan. It has been reported in Venezuela (Borges 1983), the USA (Dalmacio 2000), Japan (Nishihara 1972), Thailand (Boon-Long et al. 1988) and India (Katewa et al. 2005). The lesions caused on infected leaves are commonly reddish purple with straw colored centers, being round to elliptical and varying in size from 2 to 10 × 20 mm. The lesions in the resistant lines are small, chlorotic and clearly different from the elongated lesions on the susceptible lines. The target leaf spot fungus is also pathogenic to Sudan grass [*Sorghum sudanense* (Piper) Stapf].

To prevent the occurrence of epidemics of target leaf spot, genetic improvement of resistant varieties to the disease is considered the most effective method in sorghum. The mode of inheritance of resistance to target leaf spot in sorghum caused by *Drechslera sorghicola* (now named *Bipolaris sorghicola*) was first reported in 1979 (Borges 1979). That study showed that progeny of crosses between resistant and susceptible lines segregated for resistance in the expected ratio for resistance conferred by a single recessive gene (*ds1*). Similar results were obtained using the other cultivars in Japan (Tsukiboshi et al. 1990). Thus, the resistance to target leaf spot is controlled by a single recessive resistance gene.

Since the early 1990s, resistance genes (R genes) against various pathogens have been isolated from important crop species, such as barley, tomato and rice (Bent and Mackey 2007; Moffett 2009; Speth et al. 2007). Concept between plants and their pathogens is based on interactions between a dominant R gene in the plant against biotrophs and hemibiotrophs and a corresponding avirulence gene in the pathogen. Most such R genes resemble intracellular receptors and are characterized by a predicted Nucleotide Binding Site/Leucine Rich Repeat (NBS/LRR) structure. Other types of R genes encode a putative LRR extracellular receptor and membrane anchor, a receptor kinase with extracellular LRR ligand binding transmembrane and serine/threonine kinase domains (LRR-RK), or a serine/threonine kinase without any obvious ligand-binding domain. Among these, LRR-RKs are structurally related to the animal tyrosine and serine/threonine kinase families, with differences in their extracellular domains. LRR-RKs regulate a wide variety of developmental and defense-related processes, including host-specific as well as non-host-specific defense responses, wounding responses, and symbiosis (Torii 2004). In rice, *Xa21* is an R gene against *Xanthomonas oryzae* encoding Leucine-Rich Repeat Receptor Kinases (LRR-RKs) against *Xanthmonas oryzae* (Song et al. 1995).

Many fungi that are commonly considered necrotrophs kill host tissue and feed on the remains may actually be hemi-biotrophs, as they exhibit a biotrophic stage early in the infection process. The genetic basis of recessive resistance to necrotrophic fungal pathogens is poorly understood, and relatively few resistance genes have been characterized (Glazebrook 2005). In sorghum, the Sorghum *Pc* gene, which confers susceptibility to milo disease caused by the fungus *Periconia circinata*, is thought to be an NBS-LRR gene analog that is present in multiple tandem copies (Nagy et al. 2007). Other recessive resistance genes have been characterized for their role in plant disease establishment, including *Mlo* in barley (Piffanelli et al. 2004) and *Xa5* (Iyer and McCouch 2004), *Xa13* (Chu et al. 2004) and *Pi21* (Fukuoka et al. 2009) in rice.

Target leaf spot is caused by a necrotrophic fungal pathogen, *Bipolaris sorghicola*, and resistance to target leaf spot is controlled by a single recessive gene (*ds1*). However, the identity of this gene has not been discovered. Sorghum has a relatively small genome, twice the size of the rice genome (750 Mbp; Paterson et al. 2009). The whole genome sequence of sorghum was recently analyzed, and more than 5,000 sequence-based SSR markers derived from the genome sequence were generated to facilitate map-based cloning (Yonemaru et al. 2009).

The objective of this study was to isolate the *ds1* gene involved in target leaf spot resistance and to elucidate its function in conferring resistance. We used F_3–F_5 progeny from a cross between susceptible (bmr-6) and resistant (SIL-05) sorghum cultivars and identified the *ds1* gene by genetic mapping using sorghum SSR markers. This candidate gene encodes a receptor-like kinase with leucine-rich repeats in its extracellular domain, which is a type of protein typically associated with plant disease. Our results suggest that the downregulation or loss of function of the *ds1* gene results in resistance to target leaf spot.

Materials and methods

Plant materials

Five sorghum (*Sorghum bicolor* Moench) susceptible varieties to target leaf spot, bmr-6, BTx623, BTx624, JN43, and JN358, and five resistant to a target leaf spot, SIL-05, Nakei-MS3B, Senkinshiro, JN290EE and Greenleaf, were used. A mapping population was established from a cross between the sorghum cultivars bmr-6 and SIL-05, which are susceptible and resistant to target leaf spot, respectively. F_3 populations were grown and tested for fungal resistance at Shinshu University in Nagano, Japan, in 2007, and F_4 and F_5 populations were tested in Tsukuba, Ibaraki, Japan, in 2008 and 2009, respectively.

Field test of target leaf spot

The field test was performed using barley seeds inoculated with *Bipolaris sorghicola* BC-24 (MAFF number 511379).

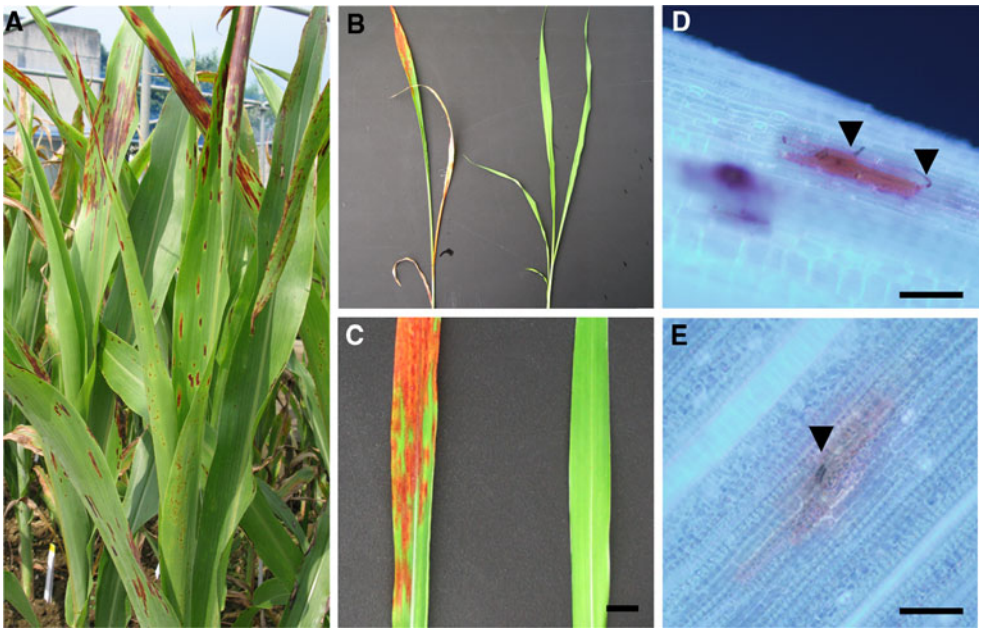

Fig. 1 Symptoms of target leaf spot in sorghum. **a** F_3 populations infected by *Bipolaris sorghicola* in test fields showed reddish spots on their leaves. **b, c** The infection test for target leaf spot in the greenhouse at the 4 leaf stage. The susceptible cultivar bmr-6 showed reddish spots, but the resistant cultivar SIL-05 did not (*right*). Black bar, 1 cm. **d, e** The infection of bmr-6 (**d**) and SIL-05 (**e**) by *Bipolaris sorghicola* at 24 h after inoculation. The fungus can infect both susceptible and resistant cultivars, and a hypersonic reaction occurred in both; however, the fungus can grow only in bmr-6. Anthocyanin accumulated intensively in bmr-6. The *arrowhead* indicates the spore of *Bipolaris sorghicola*. *Bold black bar* 100 µm

The fungus was grown for 3 weeks on sterilized barley seeds with equal amounts of water. Several seeds covered with BC-24 were placed into the sheath of sorghum plants grown in the field that were approximately 60 days of age (height ca. 40 cm). 4–7 days after inoculation, individual plants were rated for the degree of infection of their leaves on a scale from 1 to 9. The lesions on the resistant lines were small and chlorotic and clearly different from the elongated lesions on the susceptible lines (Fig. 1). The susceptible lines exhibited the purple lesions on their leaves throughout their growth.

Greenhouse seedling test for target leaf spot

Seedling tests were conducted in a greenhouse to study the reaction of the sorghum varieties and F_5 progeny of bmr-6 × SIL-05 crosses to target leaf spot. *Bipolaris sorghicola* isolate BC-24 was used as the inoculum throughout the test. The BC-24 strain was grown on vegetable juice (Campbell V8) agar for 10 days in the dark at 25°C and transferred under UV light to induce conidia for 10 days. Conidia were harvested in 0.01% Tween-20, and the concentration of suspensions was adjusted to 4×10^5 conidia/ml. At the 4–5 leaf stage, the sorghum plants were sprayed with 5 ml per pot of this suspension and grown in 1/10000a Wagnel pots (Tsukiboshi et al. 1990). The inoculated plants were kept in a moist chamber in the dark at 25°C for 16 h and then transferred to a greenhouse at 28.5–30°C. Seven days after inoculation, each individual plant was rated for the degree of infection. To evaluate the infection of BC-24, a 3-cm-long section was cut from the stems of sorghum plants at the 4-leaf stage and then inoculated with the conidia suspension in a Petri dish for 24 h. Infected spots and the accumulation of anthocyanin on the leaf samples were observed microscopically under UV light.

Marker development and genetic mapping

A mapping population was established from a cross between the sorghum cultivars bmr-6 and SIL-05. The numbers of the progeny used for genetic mapping were 175 F_3, 640 F_4, and 4235 F_5 generation plants. The sorghum simple sequence repeat (SSR) markers used for genetic mapping were designed by Yonemaru et al. (2009). The other polymorphism markers used for genetic mapping of *ds1* in the F_4 and F_5 progeny are shown in Supplemental Table 1.

Genomic DNA was isolated using a CTAB method (Murray and Thompson 1980), and specific fragments were amplified by PCR. To map the SSR markers, the 10 µl PCR mixture contained 1 U of Taq DNA polymerase (Promega), 6 nmol each of forward and reverse primers, 75 µM each dNTP and 2 ng/µl genomic DNA. PCRs were performed using 35 cycles with the following conditions: 94°C for

20 s, 55°C for 30 s and 72°C for 3 min. The resulting PCR products were analyzed by 3% agarose gel electrophoresis. The markers selected after the bulk segregation analysis were mapped in the entire population using MAPMAKER Version 3.0 (Lander et al. 1987). The F_2 intercross algorithm and default linkage criteria (LOD 3.0 and 50 cM maximum distance) were applied. The Kosambi function was used to establish genetic distances.

Construction and screening of a sorghum BAC library

Three BAC libraries were constructed from young leaves of SIL-05, bmr-6 and Greenleaf, containing 50,459 (average insert size of 128 kb), 39,267 (average insert size of 134 kb) and 55,000 (average insert size of 124 kb) clones, respectively, using conventional methods, through a partial DNA digest with the *Hin*dIII enzyme, size fractionation of high-molecular-weight DNA by pulsed-field gel electrophoresis (CHEF, Bio-Rad Laboratories), vector ligation (pIndigo BAC-5, EPICENTRE Biotechnologies) and transformation into *E. coli* (DH10B strain). Positive BAC clones covering the region of the *ds1* gene were screened from each library using tightly linked DNA markers through PCR amplification and subjected to shotgun sequencing to give approximately tenfold sequence coverage using a previously described method (Sasaki et al. 2002; Wu et al. 2003).

Two clones containing inserts from the *ds1* region, bmr6-03-P06 (171.7 kb) from bmr-6 and SIL-05-36-J06 (181.1 kb) from SIL-05, in the sorghum BACs were derived from PCR analysis using the SB12 and SB22274 PCR markers (Supplemental Table 1). The BAC sequences were produced by Sanger shotgun sequencing of subclones followed by assembly of the shotgun sequences. The sequences of candidate genes were obtained from the sorghum genome database (http://www.plantgdb.org/SbGDB) and used for gene expression analysis.

Primers were designed from the published BTx623 genome sequence to amplify overlapping fragments of approximately 4 kb that covered the entire *ds1* gene and its promoter region (Supplemental Table 2). PCR amplification was performed using Ex-Taq (Takara Bio INC, Shiga, Japan) or a high fidelity KOD-Taq (TOYOBO Co. Ltd., Osaka, Japan). The resulting PCR fragments were sequenced with the same forward and reverse primers. Sequences were assembled using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI, USA).

Phylogenetic analysis of *ds1* genes

Phylogenetic analysis using DNA sequence alignment of the *ds1* gene from various sorghum varieties and sequences deduced from the protein sequences of the LRR-RKs of a number of species were conducted with ClustalW using the neighbor-joining method (Thompson et al. 1994; Saitou and Nei 1987), and results were displayed graphically using NJ-plot (http://pbil.univ-lyon1.fr/software/njplot.html; Perriere and Gouy 1996). Support values for nodes on the tree were estimated with 1,000 bootstrap replicates. The programs used here were provided by the DNA database of Japan (DDBJ; http://www.ddbj.nig.ac.jp/search/clustalw-e.html). The LRR-RK genes used for phylogenetic analysis were as follows: *Sorghum bicolor*, Sb04g007490, Sb04g032380, Sb05g001820, Sb05g005490 (*ds1*), Sb05g004560, Sb05g004660; *Oryza sativa*, Os8g034230 (*BRI1*), Os11g0171800, Os11g0172700, Os11g0173800, Os11g0173900, Os11g0569600 (*Xa21*); *Arabidopsis thaliana*, AT3G47580, AT5G20480 (*EFR*), AT5G46330, XM_002865151 (*FLS2*); *Triticum aestivum*, ACY30448, AK334005; *Hordeum vulgare*, AK249842; *Medicago truncatula*, CU469557; *Lotus japonicus*, AP009154; *Glycine max*, AC173960; *Vitis vinifera*, XP_002268823; *Ricinus communis*, XP_002526891; *Populus trichocarpa*, XP_002317009, XP_002306327.

Gene expression analysis

The leaves of 3-month-old plants were used for expression analysis of the candidate genes. Four-leaf stage plants were used to examine the tissue specificity of the *ds1* gene and the induction of pathogen-related (PR) genes by *Bipolaris sorghicola*. For the induction of PR genes, 4-leaf stage plants were inoculated with conidia of *Bipolaris sorghicola*, and their leaves were sampled 0, 3 and 6 days after treatment. Total RNA was extracted from plant samples using the Get pure RNA Kit (Dojindo, Kumamoto, Japan). First-strand cDNA was synthesized from 1 μg of each RNA sample in a 20 μl reaction solution using the TaKaRa RNA RCR kit (AMV) Ver. 3.0 (Takara Bio Inc., Shiga, Japan). PCR was performed using 0.5-μl aliquots of cDNA solution in a 25-μl volume with AmpliTaq Gold DNA Polymerase (Applied Biosystems). The PCR profile was as follows: initial denaturation for 5 min at 94°C; 28–35 cycles of 20 s at 94°C, 30 s at 55°C, and 1.5 min at 72°C; then 5 min at 72°C for final extension. 4-μl aliquots of the PCR products were analyzed in 2% agarose gels. The PR-genes used for gene expression analysis were as follows: Pathogen related protein 1a (Agrawal et al. 2000), Sb02g002150, Sb04g035310, Sb10g001940; Chitinase (Nishizawa et al. 1999), Sb09g019660; β-1,3-Glucanase (Simmons et al. 1992); Phenylalanine ammonia lyase (PAL) (Minami et al. 1989), Sb06g022740. Sorghum actin Sb03g040880 and 18s ribosomal (Tyler et al. 2004) were used as control. The primers used for amplification are listed in Supplemental Table 3.

Results

Field and greenhouse test of target leaf spot resistance

The susceptible cultivar, bmr-6, exhibited typical stem and leaf lesions that were red to purple in color, whereas the resistant cultivar, SIL-05, was characterized macroscopically by the presence of small hypersensitive response-like chlorotic spots or no obvious symptoms. These spots were clearly different from the elongated spots on the susceptible lines (Fig. 1a–c). The susceptible lines exhibited purple lesions on their leaves throughout their growth. The conidia of *Bipolaris sorghicola* was able to infect in both resistant and susceptible lines, and no significant differences were observed in the penetration attempts between the two lines. Accumulation of phenolic compounds, presumably anthocyanins, was observed in both cultivars, but the accumulation and disease symptom were much stronger in bmr-6 (Fig. 1d, e).

Mapping of the *ds1* gene

Whole genome mapping of the *ds1* gene was performed by screening 175 F_3 plants from the cross between bmr-6 and SIL-05, and the *ds1* locus was mapped between the SSR markers SB3067 and SB3146 (not shown in 2A) on chromosome 5 (Fig. 2a). Further mapping of *ds1* was conducted by screening 229 seedlings of 640 F_4 seedlings for recombination events between two SSR markers, SB3056 and SB3178, which allowed us to narrow down the *ds1* region to a 96 kb region. Finally, we undertook high-resolution mapping of *ds1* by screening 52 seedlings of 4235 seedlings from the F_5 population for recombination events between two markers, SB12 and SB22274, and determined the location of the *ds1* locus in a 26-kb region (Fig. 2b). Both line B8-4-77 and B11-4-36 were heterozygous in this region and showed susceptible to target leaf spot. Line B11-6-7 was homozygous for the SIL-05 *ds1* region and showed resistance to the disease. In this region, only one predicted candidate locus, ORF 2 (Sb05g005490), was found in the sorghum annotation database (http://www.plantgdb.org/SbGDB/). TBLASTX searches with ORF 2 identified sequences similar to an LRR protein kinase receptor with one transmembrane domain.

Expression of target leaf spot resistance-related genes

ORF 2 was highly expressed in the leaves of the susceptible parent, bmr-6, and in the heterozygote F_5 line, but in the resistant parent, SIL-05, and F_5 plants homozygous for the SIL-05 *ds1* region, the expression of ORF 2 was extremely low (Fig. 3). These data suggest that the expression of ORF 2 is correlated with susceptibility to the disease and suppression of ORF 2 may lead to resistance to target leaf spot.

Tissue specific expression patterns of ORF 2 were analyzed in bmr-6 and SIL-05 (Fig. 4). The strong expression of ORF 2 was detected in the stem and leaves but not in roots or panicles of bmr-6 plants. In SIL-05 plants, no expression of mature RNA was detected for this gene. Unspliced mRNA was detected in the root and flower organs of SIL-05. Thus, the *ds1* gene is expressed in the aerial part of the susceptible bmr-6 plants, except in the flowers. Based on high-resolution mapping of *ds1*, the observation of a close connection between the expression of ORF 2 and the susceptibility to target leaf spot in sorghum, and the similarity of ORF 2 to LRR receptor kinase genes, which is a typical gene type associated with disease resistance and signal transduction in plants, ORF 2 is likely the *ds1* gene.

Characterization of the *ds1* gene

The BAC clones bmr-6-03P06 from the bmr-6 BAC library and SIL-36J06 from the SIL-05 BAC library were isolated and sequenced. Both BAC clones contained the *ds1* gene and its flanking region. Compared with the promoter region of BTx623 in the database, the promoter region of *ds1* in SIL-05 exhibited a 249-bp deletion following a 123-bp deletion in a 220-bp region of lower similarity (<70%) located from 2,486 to 2,975 bp upstream of the ATG codon, indicating that the deletion and substitution of this region reduced the promoter activity (Fig. 5, upper panel).

The sorghum genome annotation database predicted the *ds1* gene to contain two exons in a total sequence of 4,661 bp (3,063 bp of mRNA) coding for 1,020 amino acids (110 kDa) (Fig. 5, lower panel). Comparisons of the cDNA sequence with the genomic sequence confirmed the exon and intron boundaries. The amino acid sequence of the coding region was completely identical between SIL-05 and a susceptible variety, BTx623. There were three nucleotide substitutions in the coding sequence of the *ds1* gene in bmr-6 compared with BTx623. Two of the nucleotide substitutions are silent mutations, and the other one results in a single amino acid substitution (H851 to Q substitution). Both BTx623 and bmr-6 are susceptible varieties, and thus, both of these alleles of *ds1* encode a functional protein.

The deduced protein sequence contained one cell membrane signal peptide sequence (aa 1–24), which is presumably cleaved between C24 and S25 (SignalIP 3.0, http://www.cbs.dtu.dk/services/SignalP/; Bendtsen et al. 2004). A Pfam sequence search resulted in the identification of 10 extracellular LRR domains, a transmembrane helix (aa 636–672) and an intracellular serine/threonine kinase domain (aa 701–919) with a conserved ATP-binding motif

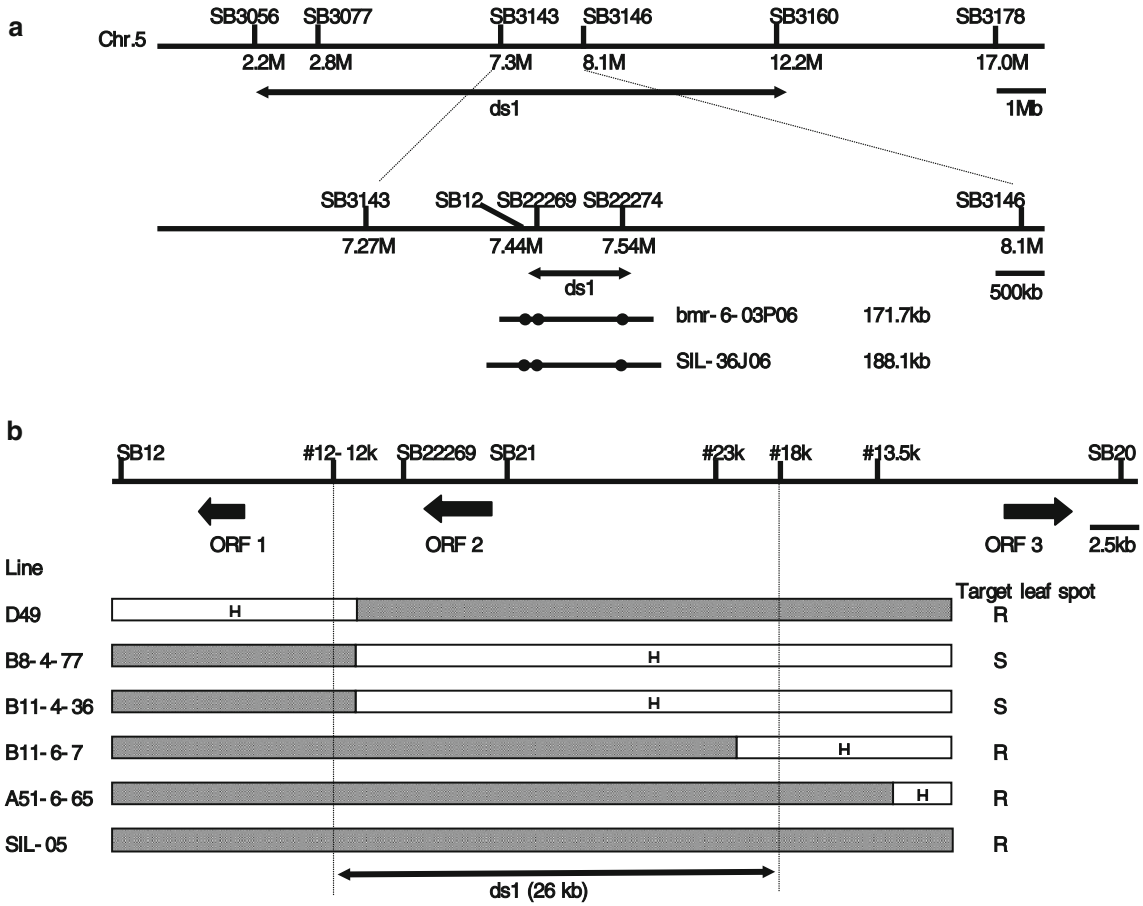

Fig. 2 Positional cloning of the *ds1* gene. **a** The *ds1* region was mapped to an approximately 10 Mb region on chromosome 5 between the SSR markers SB3056 and SB3160 in the F_3 population. Using the F_4 population, the *ds1* region was mapped to a region of approximately 96.3 kb between the markers SB12 and SB22274. The BAC clones, bmr-6-03P06 and SIL-36J06, were isolated from BAC libraries of bmr-6 and SIL-05, respectively, by PCR screening of the SB12 and SB22274 markers. **b** Using the F_5 population, the *ds1* region was mapped within a 26-kb region. The target 26-kb region containing ORF 2 (candidate for *ds1*) is shown as a *black arrow* with the flanking ORFs. The distribution of 5-single recombination events that were detected by high-resolution mapping is shown. The *hatched bar* represents the region of the SIL-05 genome, and the *open bar* represents the heterozygote region of SIL-05 and bmr-6. One ORF, ORF 2 (Sb05g005490), was found in this region from the sorghum database (http://www.plantgdb.org/SbGDB/)

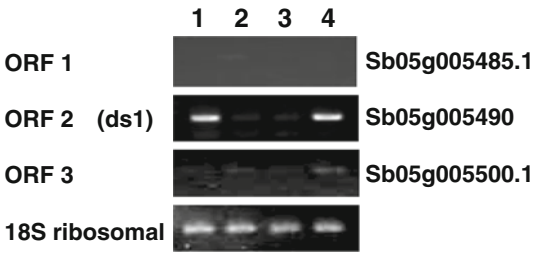

Fig. 3 Expression analyses of ORF1, -2, and -3 in the *ds1* region in a leaf. Strong expression of ORF 2 (Sb05g005490) was detected in the susceptible F_4 heterozygote (1, #2-74 A72) and in the susceptible parent, bmr-6 (*lane 4*), but not in the resistant F_4 homozygote (2, 2-74 C5) or the resistant parent, SIL-05 (*lane 3*). The expression of ORF 2 was strongly correlated with the susceptibility to target leaf spot. PCR was performed for 35 cycles for ORF genes and for 30 cycles for 18S ribosomal RNA as a positive control for RT-PCR

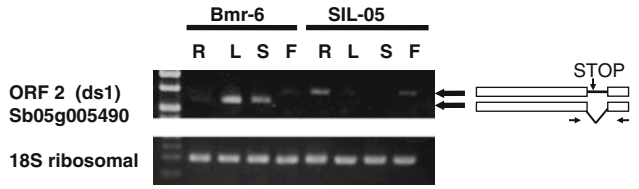

Fig. 4 Tissue specific expression of ORF 2 in bmr-6 and SIL-05. The strong expression of ORF 2 (Sb05g005490) was detected in the stem and leaves of bmr-6, but not in the roots or panicles. In SIL-05, no expression of mature RNA was detected. Unspliced mRNA was detected in the roots and flowers of SIL-05 plants. PCR was performed for 35 cycles for ORF 2 and for 30 cycles for 18S ribosomal RNA as a positive control for RT-PCR. *R* root, *L* leaf, *S* stem, *F* flower (*panicles*)

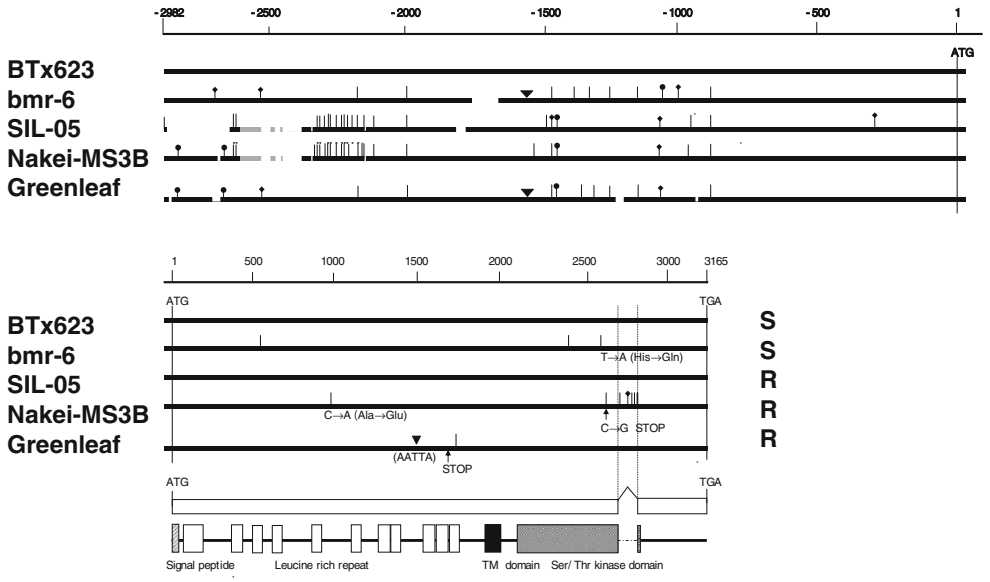

Fig. 5 Schematic diagram of the *ds1* gene (Sb05g005490) of the five sorghum varieties. *Upper panel* Schematic diagram of the promoter region of the *ds1* gene compared with BTx623. There was a deletion and a lower-homology region within 496 bp at ca. 2500 bp upstream of an ATG codon in SIL-05. *Black horizontal bar* identical sequence with BTx623, *gray horizontal bar* sequence with lower homology (approximately 70%) with BTx623, *white gap* deletion, *vertical bar* substitution of a single nucleotide, *vertical bar with black circle* insertion of a single nucleotide, *vertical bar with black square* deletion of a single nucleotide, *black inverted triangle* insertion of more than 2 bp. *Lower panel* Schematic diagram of the coding region of the *ds1* gene compared with BTx623 and the deduced protein. The *ds1* gene consists of two exons that encode a rice Xa21-like protein kinase receptor. BTx623, bmr-6 and SIL-05 encode intact proteins. Nakei-MS3B has a single point mutation that results in a stop codon. Greenleaf has a 5-bp insertion that causes a frame shift and a premature stop codon

and a serine/threonine kinase active site (http://pfam.janelia.org; (Finn et al. 2010).

Comparison of *ds1* alleles from resistant and susceptible sorghum cultivars

We compared sequences of the *ds1* gene among susceptible bmr-6 and BTx623 and resistant SIL-05, Nakei-MS3B and Greenleaf sorghum varieties (Fig. 5). In addition to the *ds1* alleles of BTx623, bmr-6 and SIL-05, two additional alleles were found in resistant varieties. Both alleles showed clear resistance to target leaf spot. The Nakei-MS3B *ds1* sequence had a C-to-G point mutation creating a UGA stop codon at aa 864 in the coding region, which would be expected to result in truncation of the kinase domain. The Greenleaf sequence had a 5-bp insertion (AATTA), causing a frame shift at codon 495, resulting in a protein of 559 aa with a different amino acid sequence.

The gene expression of *ds1* was clearly different between susceptible and resistant alleles (Fig. 6), such that it was expressed in susceptible varieties but not in resistant ones. In the case of Nakei-MS3B and Greenleaf, the transcripts were produced at a level much lower than those of susceptible lines, and mature proteins were not produced in these two varieties.

ClustalW (http://clustalw.ddbj.nig.ac.jp/top-j.html) was used to compare the base sequence of 10 cultivars and

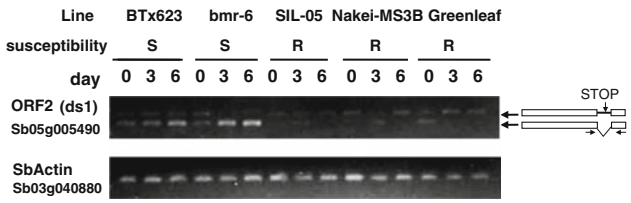

Fig. 6 The expression of the *ds1* gene after inoculation with *Bipolaris sorghicola*. The leaves were sampled 0, 3, and 6 days after inoculation. The expression of *ds1* was detected in the susceptible cultivars BTx623 and bmr-6, but not at all or in low levels in the resistant cultivars. PCR was performed for 35 cycles for ORF 2 and for 30 cycles for actin (Sb03g040880) as a positive control for RT-PCR

showed that the studied sorghum plants were grouped into 5 types based on nucleotide sequence similarity (Fig. 7). Among the varieties susceptible to target leaf spot, the sequences of BTx623 and BTx624 were identical. The sequence of bmr-6 was identical to that of JN358 and JN43 except for a single nucleotide substitution. Among the resistant varieties, the sequence of SIL-05 was identical to that of Senkinshiro and JN290EE. The resistant varieties Nakei-MS3B and Greenleaf each formed a group.

Expression of sorghum PR genes

We also analyzed the expression of pathogen related genes in bmr-6. In 3 days after inoculation, Sb02g002150 and

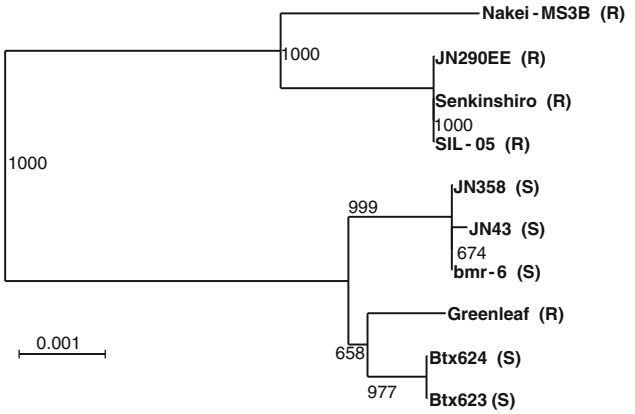

Fig. 7 Phylogenetic tree of the *ds1* gene from 10 sorghum cultivars. The promoter and coding sequences were aligned using ClustalW, and the results were displayed graphically using NJ-plot. Bootstrap values from 1,000 bootstrap replicates are shown at the nodes for assessing the robustness of the tree. The susceptibility to target leaf spot is shown in parentheses. *R* resistant, *S* susceptible

Sb10g001940 were induced, which are both homologs of the rice PR1a gene. On day 6, Sb09g019660 was slightly induced, which is a homolog of the late rice PR gene, chitinase. These results show that a pathogen related response was triggered by infection with *Bipolaris sorghicola*. However, this response was not enough to prevent the infection of bmr-6 plants.

Discussion

Resistance to target leaf spot, a disease caused by the fungus *Bipolaris sorghicola*, is conferred by a recessive resistance gene referred to as *ds1*. In this study, a candidate gene for *ds1* was identified by map based cloning. The *ds1* gene identity was confirmed by high-resolution genetic and physical mapping, sequencing of multiple alleles from resistant and susceptible lines, and gene expression analysis of the *ds1* gene. The deduced protein sequence exhibited amino acid homology to the LRR receptor kinase gene family, which encodes a leucine-rich repeat motif and a plasma membrane spanning receptor serine/threonine kinase.

Bipolaris sorghicola can infect both susceptible and resistant sorghum lines, but only susceptible lines exhibited necrotic lesions and spreading chlorosis. The induction of defense responses such as programmed cell death occurred in the susceptible variety, but this pathogen seems not to be influenced by these responses; the induction of defense responses appears to make life easier for the pathogen.

Expression of the *ds1* gene was detected in the susceptible varieties bmr-6 and BTx623. In contrast, mRNA expression was not detected or was extremely low in the resistant varieties SIL-05, Nakei-MS3B and Greenleaf. Differences in the mRNA expression of *ds1* seemed the main reason for the differences in susceptibility to target leaf spot observed in these varieties. The existence and expression of the ds1 protein resulted in susceptibility to target leaf spot. Thus, the *ds1* gene acted as a susceptibility gene in the susceptible varieties rather than a resistance gene, suggesting that loss of function of this gene leads to resistance to the disease.

In the resistant varietal SIL-05, the promoter region of *ds1* contained deletions and substitutions that may result in the reduction of promoter activity. Compared to other regions of the *ds1* gene, this region exhibited the greatest sequence variation among cultivars. In addition to the sequence polymorphisms in the promoter region, Nakei-MS3B contained a point mutation, and Greenleaf had a 5-bp insertion in the *ds1* coding region. In our expression analysis, we found that these two varieties did express ds1-related sequences (mRNA detected) but because of the frame shifts, fully functional ds1 proteins would not be produced. We analyzed the *ds1* sequences of 10 varieties and found that they could be categorized into five groups (Figs. 5, 7). The different alleles were clearly correlated with susceptibility/resistance. Interestingly, resistant Greenleaf was clustered in susceptible variety group separated from the resistant variety group in the dendrogram. The resistance of Greenleaf was caused by the 5 bp insertion of protein coding region and this insertion must have

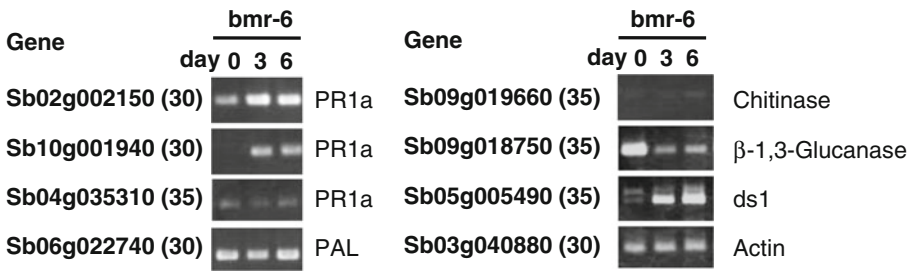

Fig. 8 The expression of pathogen related genes and the *ds1* gene after inoculation of bmr-6 with *Bipolaris sorghicola*. The infected leaves were sampled 0, 3, and 6 days after inoculation. PR proteins were induced in bmr-6, suggesting that a pathogen related reaction occurred. The numbers of PCR cycles used are shown in *parentheses*

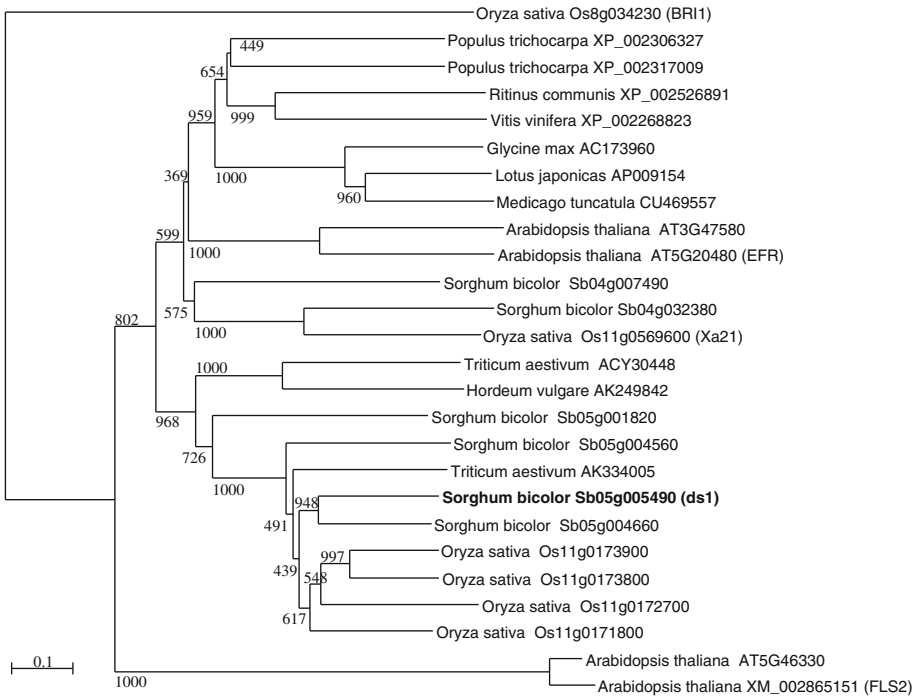

Fig. 9 Phylogenetic tree of the LRR-RKs from various plants. The protein sequences were aligned using ClustalW, and the results were displayed graphically using NJ-plot. Bootstrap values from 1,000 bootstrap replicates are shown at the nodes for assessing the robustness of the tree

happened among the susceptible lines during the domestication. These mutations causing a loss of function seemed to have occurred independently at different times.

Tsukiboshi et al. (1990) tested 42 sorghum-inbred lines for target leaf spot and found resistant lines. They studied the inheritance of the disease using four independent resistant lines and showed that F_2 progenies of the cross between resistant lines did not segregate for the susceptibility. They concluded that the resistance to target leaf spot was controlled by a single recessive gene. The F_1 hybrid, Touzankou 34, which was produced by the cross Nakei-MS3B and JN503 that has the same allele of SIL-05, showed resistance to target leaf spot. In addition, the F_1 hybrid and progenies of the cross Nakei-MS3B and Greenleaf showed resistance and did not segregate for the susceptibility (Unpublished data). Thus, the resistance in these genotypes found in this study is allelic to *ds1* and not caused by a different locus somewhere else in the genome. Until now, any exceptions for the inheritance of resistance to target leaf spot were not reported and therefore we concluded that the loss of function of the *ds1* gene resulted in resistance to target leaf spot.

The *ds1* gene encodes Leucine-Rich Repeat Receptor Kinases (LRR-RKs) and is involved in the resistance/susceptibility to target leaf spot. Three homologous genes in the same clade, Sb05g001820, Sb05g004560, and Sb05g004660, were found on chromosome 5 in BTx623 using the SALAD database (http://salad.dna.affrc.go.jp/CGViewer/; Mihara et al. 2010). They may also be involved in the resistance or defense system against diseases in sorghum. However, their function has not been determined yet. Ten homologous genes in the same clade were found on rice chromosome 11, which corresponds to sorghum chromosome 5. A QTL region related to resistance to bacterial blight was detected on the short arm of rice chromosome 11, which corresponds to the *ds1* region on sorghum chromosome 5(Wisser et al. 2005). These LRR-RKs found on rice chromosome 11 may play a role in resistance against diseases in general. However, no obvious QTLs related to plant disease are found on the corresponding region in rice (Yonemaru J-i et al. 2010). Homologous genes could be found not only in the grass family, for example, in *Oryza sativa*, *Triticum aestivum* and *Hordeum vulgare*, but also in *Arabidopsis thaliana*, *Populus trichocarpa*, *Ricinus communis*, *Lotus japonicus*, *Medicago truncatula*, *Glycine max*, and *Vitis vinifera* using an NCBI BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Fig. 9). These homologous genes may function in the plant defense system because rice Xa21 (Song et al. 1995), Arabidopsis FLS2 (flagellin-sensitive 2; Chinchilla et al. 2006) and EFR (elongation factor receptor; Zipfel et al. 2006) belong to the same class of plant receptor kinases, although their functions are still unknown.

Recently, genes required for susceptibility have been referred to as disease susceptibility genes (S-genes), including genes coding for effector targets that function as susceptibility factors or negative defense regulators (Eckardt 2002; Bent and Mackey 2007; Pavan et al. 2010). In principle, the loss of function of an S-gene, effector or host-selective toxin (HST; reviewed in Wolpert et al. 2002) target, would release the suppression of plant defense and lead to resistance. Similarly, the *ds1* gene is a recessive resistance

gene against target leaf spot, and its suppression or loss of function leads to resistance to this disease. Thus, the *ds1* gene should be considered an S-gene.

Milo disease in sorghum is caused by isolates of the soil-borne fungus *Periconia circinata* that produce PC-toxin (Macko et al. 1992). Susceptibility to milo disease is conferred by a single, semi-dominant gene, termed *Pc*. At the associated disease resistance loci, one NBS-LRR resistance gene analog, present in multiple tandem copies, was found, and it is supposed that one or more members of the NBS-LRR gene family is/are the *Pc* gene(s) that confer susceptibility (Nagy et al. 2007). In barley and *Arabidopsis thaliana*, loss-of-function mutations in *Mlo* result in efficient invasion resistance to adapted powdery mildews (Piffanelli et al. 2004; Humphry et al. 2006).

The HST victorin, which is produced by *Cochliobolus victoriae*, is active against sensitive oats and Arabidopsis. Sensitivity to victorin is clearly a dominant trait locus, referred to as *Vb* in oats and *LOV1* in Arabidopsis. The *LOV1* gene encodes a Coiled Coil-Nucleotide Binding site Leucine Rich Repeat (CC-NB-LRR) gene, which encodes a type of protein that is typically associated with plant disease (Sweat and Wolpert 2007).

Rice *Xa5* and *Xa13* are also considered S-genes, and they encode the gamma subunit of transcription factor IIAγ and a plasma membrane-localized protein, respectively (Iyer and McCouch 2004; Chu et al. 2004). Their loss of function induces resistance to bacterial leaf blight caused by *Xanthomonas oryzae*. Rice *pi21* is another recessive resistance gene against rice blast disease, caused by *Magnaporthe oryzae*, that was recently identified (Fukuoka et al. 2009). *pi21* encodes a proline-rich protein that includes a putative heavy metal-binding domain and putative protein-protein interaction motifs, and the suppression of this gene increases resistance.

The *ds1* gene is structurally related to the rice *Xa21* gene (Os11g0569701), which is known as an R gene of the LRR-RKs against *Xanthomonas oryzae* (Song et al. 1995) The structure of the ds1 protein suggests a role in cell surface recognition of a pathogen ligand or HSTs and subsequent activation of an intracellular defense response. Several studies have indicated that LRR-RKs act as heterodimers, like the BAK1 protein, and some may form a receptor complex with leucine-rich repeat receptor-like proteins (Li et al. 2002; Ali and Reddy 2008; He et al. 2008; Zipfel 2008) . In the case of ds1, intermolecular interactions probably exist that result in the formation of a protein complex. The difference of components of the complex may alter signal transduction and the Hypersensitive Response (HR) due to interaction with the ds1 protein, which may alter susceptibility to the disease.

In our experiments, we did not detect any additional QTLs for resistance to target leaf spot. However, other genes must be involved in the progression of target leaf spot as secondary factor since the rate of disease progression is different between susceptible varieties. Genetic mapping between susceptible varieties and detailed expression may help to identify differences in the HR response. PR proteins are induced in bmr-6 (Fig. 8), suggesting that the HR response leads to cell death and promotes the *Bipolaris sorghicola* infection. It is interesting to suppose that there is a specific ligand or HST interaction with the ds1 protein produced by *Bipolaris sorghicola*, such as a peptide for Xa21 (Lee et al. 2009), flagellin (flg22) for FLS2 (Chinchilla et al. 2006) and elongation factor-Tu for EFR (Zipfel et al. 2006). However, such molecules have not been identified yet, and this question remains to be solved.

In practice, the majority of resistance breeding programs have aimed to introduce R-genes from a resistant species into susceptible crop plants, but such resistance can be overcome by pathogens, resulting in outbreaks of large epidemics. However, loss of function of S-genes often leads to stable and broad-spectrum resistance. The stability of a resistance gene needs to be proven when cultivars carrying that gene alone maintain prolonged resistance under natural field conditions (Hammond-Kosack and Parker 2003; Pavan et al. 2010). Thus, this type of disease resistance is of significance for durability of resistance during crop domestication. So far, race-specificity of resistance against *Bipolaris sorghicola* has not been found. In addition, we have not found any other phenotypes associated with loss-of-function of the ds1 protein. The plants with loss of function alleles look as the same as those of functional alleles morphologically and they grow quite normal and healthy. Therefore, using loss of function alleles of the *ds1* gene, we can breed new sorghum cultivars with stable and broad-spectrum resistance in the future.

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