Theor Appl Genet (2012) 124:1005–1015
DOI 10.1007/s00122-011-1764-8

ORIGINAL PAPER

Two loci in sorghum with NB-LRR encoding genes confer resistance to *Colletotrichum sublineolum*

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Received: 21 September 2011 / Accepted: 23 November 2011 / Published online: 6 December 2011

Abstract The aim of this work was to identify plant resistance genes to the sorghum anthracnose fungus *Colletotrichum sublineolum*. cDNA-AFLP transcript profiling on two contrasting sorghum genotypes inoculated with *C. sublineolum* generated about 3,000 informative fragments. In a final set of 126 sequenced genes, 15 were identified as biotic stress related. Seven of the plant-derived genes were selected for functional analysis using a *Brome mosaic virus*-based virus-induced gene silencing (VIGS) system followed by fungal inoculation and quantitative real-time PCR analysis. The candidate set comprised genes encoding resistance proteins (*Cs1A, Cs2A*), a lipid transfer protein (*SbLTP1*), a zinc finger-like transcription factor (*SbZnTF1*), a rice defensin-like homolog (*SbDEFL1*), a cell death related protein (*SbCDL1*), and an unknown gene harboring a casein kinase 2-like domain (*SbCK2*). Our results demonstrate that down-regulation of *Cs1A*, *Cs2A*, *SbLTP1*, *SbZnF1* and *SbCD1* via VIGS, significantly compromised the resistance response while milder effects were observed with *SbDEFL1* and *SbCK2*. Expanded genome analysis revealed that *Cs1A* and *Cs2A* genes are located in two different loci on chromosome 9 closely linked with duplicated genes *Cs1B* and *Cs2B*, respectively. The nucleotide binding-leucine rich repeat (NB-LRR) encoding *Cs* gene sequence information is presently employed in regional breeding programs.

Communicated by A. Paterson.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-011-1764-8) contains supplementary material, which is available to authorized users.

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Introduction

In all natural environments, plants are rarely able to grow without attempted pathogen colonization and have evolved both passive and active mechanisms to prevent infection. Active defense mechanisms are based upon an early recognition of the intruder followed by appropriate responses. Like animals, plants have evolved two distinct categories of immune receptors, each of which recognizes non-self molecules (reviewed in Chisholm et al. 2006; Cui et al. 2009). The first class, which contributes to basal defense, involves membrane-resident pattern recognition receptors that detect conserved microbe-associated molecular patterns (MAMPs) such as bacterial flagellin or fungal chitin. This first line of defense against pathogens is reminiscent of innate immunity in vertebrates. The expression of basal resistance to invasive pathogens is a crucial protective defense barrier. Without it, plants become susceptible to even mild infections and are less likely to survive in a competitive environment. The second strategy is effector-triggered immunity (ETI) represented by resistance (R) proteins, which are mainly intracellular, having the capacity to directly or indirectly detect isolate-specific pathogen effector proteins, encoded by avirulence (*Avr*) genes. Variants of

ETI responses are emanating from other pathosystems than the well-studied *Pseudomonas syringae–Arabidopsis thaliana* interaction (Jones and Dangl 2006; Cunnac et al. 2009; Hein et al. 2009; Deller et al. 2011). Unlike the situation in mammalian adaptive immunity, the plant host-specific determinants of effector-triggered immunity are encoded in every cell of an organism. Detection of these effectors trigger signaling cascades that lead to diverse cellular responses such as activation of mitogen-activated protein kinase signaling and various defense genes via transcription factors or defense signaling mediated by hormones (Bari and Jones 2009; Staal and Dixelius 2009).

Maize (*Zea mays*) and sorghum (*Sorghum bicolor*) are the most important staple cereals for sub-Saharan Africa (SSA). While maize is an introduced crop (Mangelsdorf 1974), sorghum is believed to have been domesticated in SSA, presumably in the Nile basin or Ethiopia as recently as 1000 BC (Kimber 2000). Sorghum together with maize and sugarcane form a closely related phylogenetic group compared to other grasses like rice, wheat and barley (Paterson et al. 1995). Rice, sorghum and maize now have their genomes sequenced (Paterson et al. 2009; Schnable et al. 2009). Sorghum is generally cultivated in drier and marginal regions of the African continent but with low grain yield. Due to its high drought tolerance properties it is now a highly prioritized crop since rainfall patterns have become more unpredictable (Sasaki and Antonio 2009; Olembo et al. 2010).

Sorghum like many other crop species experience large problems with plant pathogens, particularly fungal diseases. Sorghum anthracnose incited by *Colletotrichum sublineolum* is one of the main problems (Thomas et al. 1996; Ngugi et al. 2002). Reports on defense genes and mechanisms to this fungal pathogen are fragmentary and partly confounded since *C. sublineolum* to a certain degree has been mistaken as *C. graminicola*, the maize stalk rot pathogen (Sutton 1980; Pastor-Corrales and Frederiksen 1980). In order to avoid this confusion, taxonomy of *Colletotrichum* species is now based on molecular data (Hsiang and Goodwin 2001). Resistance gene loci to *C. sublineolum* in sorghum have earlier been denoted *Cg* reflecting the former connection with *C. graminicola* (Perumal et al. 2009). In this work, plant resistance loci and genes are named *Cs* to avoid further confusion. Typical symptoms of foliar sorghum anthracnose infection appear as circular spots or elongated lesions with purple, red, tan, or black margins depending on host plant pigmentation (Frederiksen 1989; Thakur and Mathur 2000; TeBeest et al. 2004). Black, asexual fruiting bodies, acervuli (aggregated hyphal structure which erupt through the epidermis, characteristic of *Colletotrichum* spp.), appear in the center of the lesions during sporulation. Coalescence of lesions is typically observed on susceptible varieties and may result in leaf senescence and plant death. Rapid cell death in local spots on the sorghum leaves is a common feature of the resistance reaction. Sorghum plants can also accumulate a range of phenolic substances in response to pathogen attacks (Dicko et al. 2005; Liu et al. 2010). In case of *C. sublineolum*, orange–red flavonoids are often clearly visible in both compatible and incompatible host plant reactions depending on genomic background (Lo et al. 1999a; Yu et al. 2005). The capacity of anthocyanin accumulation is generally reflected by seed color, i.e., red seed leads to high levels of anthocyanins in the plant (Ibraheem et al. 2010).

In this work, we explored East African sorghum genotypes using a comparative cDNA-amplified fragment length polymorphism (AFLP) analysis followed by functional assessment of selected gene candidates by virus-induced gene silencing (VIGS) using a *Brome mosaic virus* vector. We found seven defense-related gene candidates whereof two NB-LRR encoding genes residing in two different loci on chromosome 9.

Materials and methods

Plant and fungal materials

Two contrasting *Sorghum bicolor* genotypes from Uganda, BS04/05 and MU07/193D resistant and susceptible to *C. sublineolum*, respectively, were used in the study. The plants were grown in a growth chamber (Percival, IA, USA) using a 12/12-h photoperiod at 22°C. The fungal isolate used was isolated from naturally infected sorghum in Uganda. After single spore isolation, the fungal DNA was analyzed by using ITS1 and ITS2 primers (F-GATAACCAAACTCTGATTTAACGA and R-GGTCAACCAGTTAAAAGTTAGGG) as outlined by Hsiang and Goodwin (2001). PCR amplification was run as following: 3 min at 94°C, 35 cycles of (1 min at 94°C, 1 min at 60°C, and 1.5 min at 72°C), and final extension at 72°C for 10 min. The PCR products were separated on 1% agarose gels to confirm fragment size (376 bp), followed by sequencing, generating 98–100% sequence match.

Inoculation of plant material

Three-week old BS04/05 and MU07/193D sorghum seedlings were spray inoculated on the third leaf with conidia suspension (5×10^6 conidia/ml) as described by Lo et al. (1999b). The inoculated plants were covered with plastic film for 24 h to ensure high humidity. Inoculated leaves from three to four plants were pooled and harvested at 24, 48, and 78 h post-inoculation (hpi) for cDNA-AFLP analysis. Water-treated control samples were harvested at the same time points.

RNA extraction and cDNA-AFLP analysis

Total RNA was isolated from the leaf samples using the BioRad RNA isolation kit (BioRad, California, USA) followed by mRNA preparation with the mRNA capture kit (Roche, CA, USA). cDNA was synthesized with Oligo-dT primer and RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas, Helsingborg, Sweden). Second strand was synthesized using *E. coli* DNA Polymerase I (Fermentas). The double stranded cDNA was digested with *Bst*Y1 and *Mse*1 (Fermentas) and ligated to respective adaptors, pre-amplified and later selectively amplified using the *Bst*YI +N (^{33}P labeled) and *Mse*I +N primers. Pre-PCR amplification was carried out with the adapter-ligated cDNA, Taq DNA Polymerase (Fermentas) and the non-selective primers specific to the *Bst*YI and *Mse*I adapters using 25 cycles of 94°C for 30 s; 56°C for 1 min and 72°C for 1 min. The pre-amplified reaction mixture was diluted 600-fold and 5 µl was used for final selective amplification with 24 primer combinations, carried out with *Bst*YI +N (^{33}P labeled) primers (ESM Table S1) and touchdown amplification (Vos et al. 1995). The selective amplification products were resolved on 6% polyacrylamide gel run at 100 W until 4,300 Vh was reached. Gels were dried and exposed to Kodak Biomax film (Amersham Pharmacia, CA, USA) for 5–7 days.

Isolation and sequencing of transcripts

Approximately 160 transcripts (unique, up- and down-regulated) from the resistant genotype in relation to the susceptible genotype were excised from the dried PAGE gels, eluted in H_2O and PCR amplified using the non-selective primers under the same conditions as earlier described in the pre-amplification step. The products were cloned into the pJET 1.2 blunt vector (CloneJET™ PC, Fermentas) and sequenced (Macrogen Inc., Seoul, Korea). The sequences were analyzed using the BLASTN and BLASTX programs (Altschul et al. 1997) and compared with sequences deposited in NCBI, GRAMENE and PHYTOZOME databases. A short list of 126 genes was generated (ESM Table S2) from which seven candidate genes were selected for further analysis. The gene candidates were predicted to encode; a nucleotide binding site (NB) and a leucine rich repeat (LRR) motif (*Cs1A*; Sb09g027470), a coiled coil (CC), NB-LRR and a WD40 domain at the C-terminal end (*Cs2A*; Sb09g004240), a lipid transfer protein (Sb10g021170), a zinc finger-like transcription factor (Sb03g041170), a rice defensin-like homolog (Sb01g017080), a cell death related protein (Sb09g027280), and an uncharacterized gene (Sb03g036189) with a casein kinase 2 domain.

VIGS in sorghum

The VIGS system used is based on the monocot-infecting *Brome mosaic virus* (BMV) as previously described (Ding et al. 2006), but pre-inoculation on barley was excluded as intermediate virus host. The BMV VIGS vector consists of a combination of three plasmids harboring BMV RNA1 (p1-1), RNA2 (p2-2) and RNA3 (pF13m, previously pF3-5/13$_{A/G}$), respectively. To generate VIGS constructs, PCR fragments ranging from 247 to 392 bp in size were amplified from the sorghum candidate genes using genomic DNA of the resistant BS04/05 genotype and gene-specific primers harboring *Nco*I and *Avr*II restriction sites (ESM Table S3). Prior to PCR amplification, off-target gene searches were undertaken to optimize gene targeting. After restriction, each fragment was cloned into the corresponding site of the pF13m plasmid. The identity of the inserts was verified by sequencing. The three plasmids were digested with *Spe*I, *Psh*AI and *Psh*AI, respectively. Infectious RNA transcripts were synthesized from linearized plasmids through in vitro transcription. Inoculation procedures were performed as described (Ding et al. 2006) with slight modifications. A 10-µl aliquot of the transcription mix from each of the plasmids p1-1, p2-2 and pF13m-insert was combined with 30 µl of the inoculation buffer and used directly to rub inoculate the second and third leaves of 3-week-old sorghum plants. As a control, sorghum plants were inoculated in the same way with combined transcripts from p1-1, p2-2 and empty pF13m. The plants were challenged with *C. sublineolum* as earlier described 2 weeks after viral inoculation (when faint chlorosis and vein clearing started to appear) to assess the effect of the different constructs on the sorghum–*C. sublineolum* interaction. The choice of infecting the second and third leaves was based on a pre-study where a 237-bp antisense fragment of the *phytoene desaturase* (*PDS*) gene from wheat (PCR primers, see EMS Table S3) was inserted to the BMV pF13m plasmid. Plants inoculated with the pF13m-PDS construct showed a white streaky phenotype on the second and third leaf above the inoculation site indicative of PDS silencing (data not shown).

Quantitative real-time PCR (qRT-PCR)

Prior to fungal inoculation, approximated 3 cm of VIGS inoculated leaves were collected from three plants, forming one sample and used for RNA extraction as previously described. Four biological samples were analyzed per VIGS construct, and the resistant wild-type BS04/05 (control). First strand cDNA was synthesized from 1 µg of total RNA, with Oligo-dT primer and RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas) according to the manufacturer's instructions. Real-time PCR was carried out using the first strand cDNA in an iQ5 cycler (Bio-Rad). The DyNAmo™ ColorFlash SYBR Green qPCR Master Mix Kit (Finnzymes,

Espoo, Finland) was used for PCR amplification in a 20-µl total reaction volume consisting of 10 µl of SYBR Green qPCR Master Mix, 3 µl of forward and reverse primers and 5 ng of cDNA template. All PCRs were performed in triplicate under the following amplification conditions; 15 min at 95°C followed by 40 cycles of 15 s at 95°C and annealing/extension at 60°C for 1 min followed by a melt curve analysis. Primers sequences for the seven target genes were designed using the Primer 3 version 0.4.0 (http://frodo.wi.mit.edu/primer3/) software (ESM Table S4). The sorghum *SbACTIN* (*Sb01g010030*) and 18S rRNA genes were used for normalization of expression levels (Yang et al. 2004; Shen et al. 2010). All calculations and statistical analyses were performed as described in the ABI PRISM 7700 Sequence Detection System User Bulletin #2 (Applied Biosystems, USA) slightly modified by Vetukuri et al. (2011).

Genome analysis

The sorghum genome was searched using BLASTP and BLASTN with the seven candidate genes as query in order to identify any other gene family members. In the case of *Cs1A* and *Cs2A*, a second gene member, *Cs1B* and *Cs2B*, respectively, was found in close proximity on chromosome 9. DNA and amino acid sequences of Cs1A (Sb09g027470), Cs1B (Sb09g027520), Cs2A (Sb09g004240), and Cs2B (Sb09g 004210) were aligned to sorghum, maize, millet, rice, *Brachypodium* and *Arabidopsis* genome databases using BLASTN and BLASTP (Altschul et al. 1997) and PHYTOZOME. Homologs from *Arabidopsis* and rice were aligned using CLUSTALW (Larkin et al. 2007). Identical and similar residues among sequences were highlighted using the Boxshade program 3.2 (http://www.ch.embnet.org/software/BOX_form.html). Predicted domains were identified using coiled-coil prediction (Lupas et al. 1991), LRRfinder (Offord et al. 2010) and CD-Search (Marchler-Bauer and Bryant 2004). Genes in close proximity to *Cs1A* and *Cs2A* were identified using Genomic Viewer (PHYTOZOME). Phylogenetic analysis was conducted using Treefinder and maximum likelihood and 10,000 replicates (Jobb et al. 2004). The JTT+G model (Jones et al. 1992) was found to best fit the data using ProtTest v2.4 (Abascal et al. 2005). Confidences were calculated using local rearrangement of expected likelihood (LR-ELW) (Strimmer and Rambaut 2002). Phylograms were drawn using Treeview 1.6.6 (Page 1996).

Results

cDNA-AFLP identification of stress-related transcripts

In order to identify important plant defense genes in the sorghum–*C. sublineolum* interaction, cDNA-AFLP analysis was carried out on two contrasting sorghum genotypes, the resistant BS04/05 and the susceptible MU07/193D, carefully selected from an East African germplasm collection (Biruma, in preparation). In total, approximately 3,000 transcript-derived fragments (TDFs) were monitored ranging from 50 to 600 bp in size. Unique, up- or down-regulated transcripts in BS04/05 genotype compared to MU07/193D sorghum line, sampled at 24, 48 and 72 h post-inoculation (hpi) were excised, sequenced and analyzed for predicted function (ESM Table S2). The final TDF-set comprising 126 gene candidates deriving from the resistant BS04/05 genotype contained 25 sequences that were unique (being highly expressed only in the resistant BS04/05 genotype), 94 pathogen up-regulated and 32 pathogen down-regulated transcripts. Fifty-two transcripts were up-regulated in the resistant genotype at 24 hpi while 30 and 12 were up-regulated at 48 and 72 hpi, respectively. Based on known or predicted functions in other plant species, 15 genes could be identified as biotic stress related (ESM Table S4). The relative transcript levels identified in the cDNA-AFLP analysis were further validated by qRT-PCR performed on selected 14 up- and down-regulated genes in the resistant genotype 24 hpi (Fig. 1).

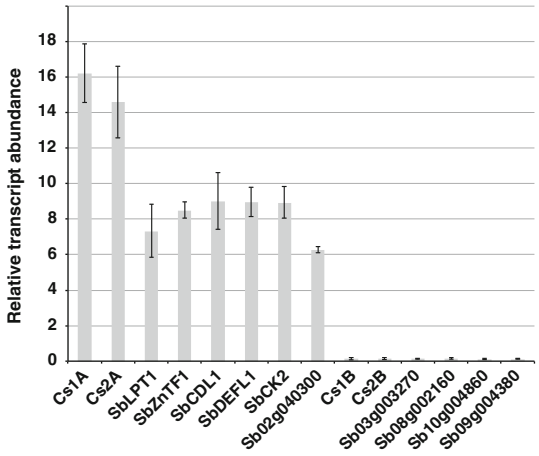

Fig. 1 Relative transcript levels of 14 sorghum genes in *C. sublineolum*-inoculated BS04/05 genotype (resistant). The expression at 24 h post-inoculation was analyzed by real-time PCR. The relative levels were calculated with respect to the corresponding transcripts in uninfected (water-treated) plants. *Bars* indicate standard deviation between three biological replications

Functional analysis of putative defense genes

In order to evaluate possible involvement in the plant defense mechanism, seven strongly up-regulated transcripts in the resistant BS04/05 genotype compared to the susceptible MU07/193D line, were selected based on their predicted function, expression profile as revealed by reverse-transcription (RT)-PCR and qRT-PCR, and low copy number in the

genome. The selected gene candidates were predicted to encode; a nucleotide binding site (NB) and a leucine rich repeat (LRR) motif (*Cs1A*; Sb09g027470), a coiled coil (CC), NB-LRR and a WD40 domain at the C-terminal end (*Cs2A*; Sb09g004240), a lipid transfer protein (Sb10g021170), a zinc finger-like transcription factor (Sb03g041170), a rice defensin-like homolog (Sb01g017080), a cell death related protein (Sb09g027280), and an uncharacterized gene (Sb03g036189) with a casein kinase 2 domain. These seven gene candidates are, in the order given above, denoted resistance to *C. sublineolum Cs1A* and *Cs2A*, together with *SbLTP1*, *SbZnTF1*, *SbDEFL1*, *SbCDL1* and *SbCK2*.

Genetic transformation of sorghum is possible but laborious (Gurel et al. 2009), and requires genotypes other than those used in this study to be successful. Hence, all candidate genes were further studied using VIGS followed by fungal inoculation to assess their potential defense function. The VIGS procedure was first optimized. Sorghum seeds were surface sterilized before sowing to minimize additional stress by other microorganisms. The seedlings were thinned out to generate homogeneous stands and humidity was kept high in the later phase to ensure successful fungal establishment in the plants. mRNA was produced by in vitro transcription, added to inoculation buffer and rubbed directly onto the second leaf of 3-week-old sorghum plants. Fungal growth on plants inoculated with the different VIGS constructs compared with control material was carefully monitored. To this end, potential variation among plants was minimized by scoring repeated plant sets grown in culture cabinets with a randomized design and coding the plants to avoid biased scorings.

The *Cs1A* and Cs2*A* genes have very low similarity on DNA sequence level in contrast to *Cs1A* and *Cs1B* (81% identity), and *Cs2A* and *Cs2B* (82% identity). Consequently, cross-silencing between the two loci was not possible to carry out. However, the *Cs1A* and *Cs2A* VIGS constructs were designed to also target the *Cs1B* and *CS2B* genes, respectively.

Fungal colonization and growth on plants inoculated with the different VIGS constructs compared with control material was carefully monitored. The phenotypic observations are summarized in Table 1 and leaf phenotypes shown in Fig. 2 and in EMS Fig. S1. A typical hypersensitive response (HR) similar to the resistance response in the wild type was observed when BMV empty vector was applied followed by fungal inoculation (Fig. 2a, i) indicating that the vector does not compromise the immune system of the plant. Among plants pre-inoculated with the different gene constructs, variations were observed in respect to time of onset of lesion formation, lesion size and spread, and formation of acervuli with conidiophores suggesting that the seven genes examined are of more or less importance in this specific defense system. Empty vector control and down-regulated genes (Fig. 1) did not impact the phenotypic response when tested in the VIGS system (Fig. 2; data not shown). From the phenotypic data assembled it became apparent that down-regulation of *Cs1A, Cs2A, SbLTP1, SbZnF1* and *SbCD1* on the resistant sorghum genotype significantly compromised the resistance response to *C. sublineoum*. This observation was somewhat different to *SbDEF1* and *SbCK2* plants which resulted in a weak susceptible phenotype upon virus-induced gene silencing. In order to compare the observed VIGS-related phenotypes with attempted target gene transcript levels, qRT-PCR was carried out on leaf samples from the VIGS experiments (Fig. 3). There was a significant decrease in the relative transcript levels in relation to control plants inoculated with empty vector suggesting a down-regulation of all targeted genes. The transcript levels of *Cs1B* and *Cs2B* genes were also affected as expected.

The two sorghum genotypes used in the study have red seed color, a trait that is known to impact disease phenotypes due to flavonoid accumulations in leaf tissue (Yu et al. 2005). Consequently, large amounts of red-pigmented flavonoids were induced at the site of colonization, particularly upon the *C. sublineolum* compatible interaction. The strong anthocyanin response in the plant material excluded detailed cytological examinations, staining or otherwise close-up microscopic analysis of fungal growth and colonization on the leaves.

The Cs2 proteins have uncommon domain architecture

Genome analysis revealed that *Cs1A* and *Cs2A* genes are located in two different loci on chromosome 9 closely linked with duplicated genes *Cs1B* and *Cs2B*, respectively (Fig. 4). Both *Cs1A* and *Cs2A* genes were strongly up-regulated by fungal challenge in the *C. sublineolum* resistant sorghum genotype examined (Fig. 1). Transcription of *Cs1B*, *Cs2B*, *Sb09g004215*, *Sb09g004220* and *Sb09g004230* were further examined in the resistant BS04/05 genotype. *Cs1B* and *Cs2B* generated faint bands upon RT-PCR analysis. Similarly, very low relative transcript levels, less than 0.2 compared to corresponding transcripts in water-treated plants were generated via qRT-PCR. None of the additional three genes between the *Cs2* duplicates (Fig. 4) produced any product upon RT-PCR analysis. The Cs1A protein lacks the predicted CC domain and is shorter than Cs1B. A sequence comparison of Cs2A and Cs2B shows further that Cs2A has a shorter CC domain, and both Cs2A and Cs2B proteins were predicted to harbor a WD40 domain at their C-terminal ends. This WD40 motif with the feature of a ~40 amino acid stretch typically ending in Trp–Asp, was confirmed by re-sequencing additional cDNA samples. When searching genome databases, a single homologous protein to Cs2A and Cs2B with all four predicted domains

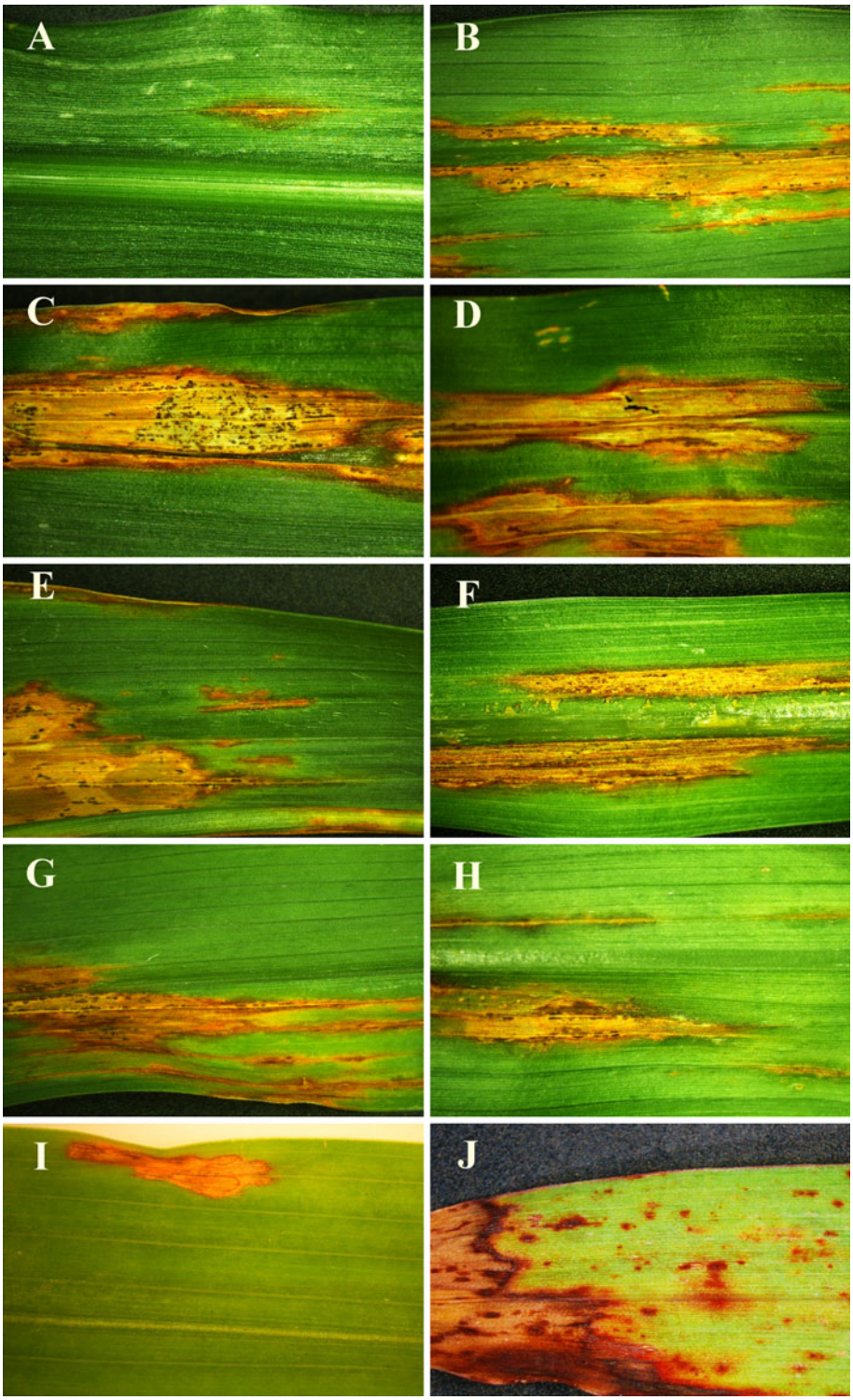

Fig. 2 Disease phenotypes on sorghum leaves observed 12 days post-inoculation with *C. sublineolum* on the resistant BS04/05 genotype pre-inoculated with the BMV vector with constructs with following genes: **a** Empty BMV vector; **b** *Cs1A*; **c** *Cs2A*; **d** *SbLTP1*; **e** *SbZnTF1*; **f** *SbDEFL1*; **g** *SbCDL1*; **h** *SbCK2*; **i** No BMV vector, only fungal inoculation; **j** No BMV vector, susceptible MU07/193D genotype fungal inoculated

was only found in rice (ESM Fig. S2 and Fig. S3). Rice belongs to the subfamily Ehrhartoideae while sorghum and maize belong to the subfamily Panicoideae (Bolot et al. 2009). These two sub-families among the grass species are rather divergent since the evolutionary split between ancestors took place 50–70 million years ago. Some homology was also found between Cs2A and Cs2B and *Arabidopsis* CC-NB-LRR proteins. The nearest related genes are *RPP13* that confer resistance to *Hyaloperonospora parasitica* (Bittner-Eddy et al. 2000) and *RPM1*, a gene mediating resistance to *Pseudomonas syringae* isolates expressing the *avrRpml* or *avrB* genes (Grant et al. 1995) (Fig. 5; ESM Fig. S2). The clustering of the two Cs2 proteins together with a number of characterized R-proteins in *Arabidopsis*,

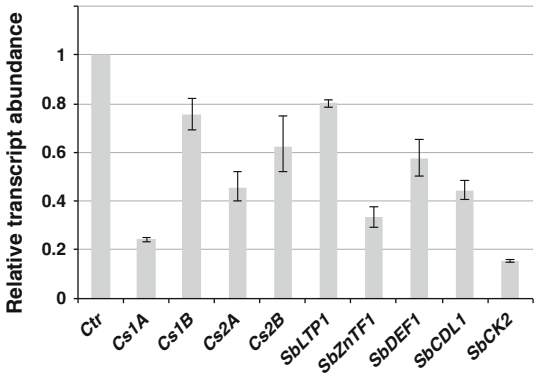

Fig. 3 Real-time PCR analysis of the silencing response obtained in sorghum (resistant BS04/05 genotype) following inoculation with different VIGS gene constructs. Leaf samples were taken immediately prior to the time of fungal inoculation. Plants inoculated with an empty BMV vector were taken at the same time and used as control (Ctr). *Bars* indicate standard deviation between three biological replications

further suggest a more ancient evolutionary history compared to the proteins in the *Cs1* locus (Fig. 5, ESM Fig. S3 and S4). Similar genome events have occurred among the CC-NB-LRR encoding genes in sorghum effective to *Setosphaeria turcica*, a fungus causing turcicum leaf blight, that reside in three pairs, in one locus, on chromosome 5 (Martin et al. 2011). The most similar but very distant related *Arabidopsis* gene to *Cs1A* and *Cs1B*, is *TAO1* (target of AvrB operation) (Fig. 5), a TIR (Toll-interleukin 1 receptor) -NB-LRR encoding gene (Eitas et al. 2008) with BLASTP similarities of $1e^{-08}$ and $5e^{-06}$, respectively. In order to find related grass proteins, the top BLASTP hits together with Cs1A and Cs1B were used to create a phylogram (ESM Fig. S4.) Cs1A and Cs1B cluster separately from related proteins in other grass species and also other NB-LRR encoding genes in sorghum. This indicates that *Cs1A* and *Cs1B* are the result of a recent duplication event after the split of sorghum and maize ~10 million years ago.

Discussion

In this work we identified seven genes in sorghum that contribute to defense to *C. sublineolum* causing sorghum anthracnose. Particularly the NB-LRR encoding *Cs* genes attracted our attention. Two genes, *Cs1A* and *Cs2A* were found based on cDNA-ALFP data. They are located in two separate loci on chromosome 9. Closer studies of sequences in each locus revealed presence of additional *Cs* genes. Both *Cs* gene pairs, i.e., *Cs1A* and *1B*, and *Cs2A* and *2B*, respectively, were targeted in the VIGS analysis and shown to contribute to the resistance response.

The VIGS method became highly reliable after our modifications and use of growth cabinets with controlled conditions. To generate enough material for qRT-PCR analyzes, we were forced to pool leaf samples excluding analysis of individual leaves or plants. This practice may have influenced the qRT-PCR data, together with factors such as timing of sampling and onset of the defense response and associated signaling cascades. Because the qRT-PCR values are high in the resistant genotype BS04/05 compared to the susceptible MU07/193D line, including preliminary genetic data linking disease resistance with presence of the *Cs* genes, we have concluded that the *Cs1* and *Cs2* gene pairs are the major plant defense components in our materials.

A wealth of information about NB-LRR genes is provided by the public available whole genome sequences. In the *S. bicolor* genome, 211 NB-LRR encoding R-proteins are present, which are approximately half the number

Table 1 Disease phenotypes on sorghum leaves, monitored 1–12 days post-inoculation (dpi) with *C. sublineolum* on the resistant wild-type BS04/05 genotype, and BS04/05 pre-inoculated with: empty BMV vector, *BMV::Cs1A, BMV::Cs2A, BMV::SbLTP1, BMV::SbZnTF1, BMV::SbCDL1*, *BMV::SbDEFL1*, and *BMV::SbCK2*

Plant material	HR-response	Lesion occurrence	Lesion size (cm) 10 dpi	Lesion spread	Acervuli formation 12 dpi
Resistant BS04/05	Yes	No	No	No	No
Empty BMV vector	Yes	No	No	No	No
BMV::Cs1A	No	7 dpi	5–8	Coalesced 12 dpi	Moderate
BMV::Cs2A	No	7 dpi	5–8	Coalesced 12 dpi	Profuse
BMV::SbLTP1	No	6 dpi	7–10	Coalesced 10 dpi	No
BMV::SbZnTF1	No	7 dpi	5–9	Coalesced 10 dpi	A few
BMV::SbCDL1	No	7 dpi	5–9	Coalesced 11 dpi	A few
BMV::SbDEFL1	No	9 dpi	3–6	Minimal coalescence 12 dpi	No
BMV::SbCK2	No	9 dpi	3–6	Minimal coalescence 12 dpi	No
Susceptible MU07/193D	No	4 dpi	8–11	Coalesced 10 dpi	Profuse

The MU07/193D genotype was included as a susceptible control. The data is compiled from 25 to 30 plants per BMV construct and controls. The experiment was repeated three times

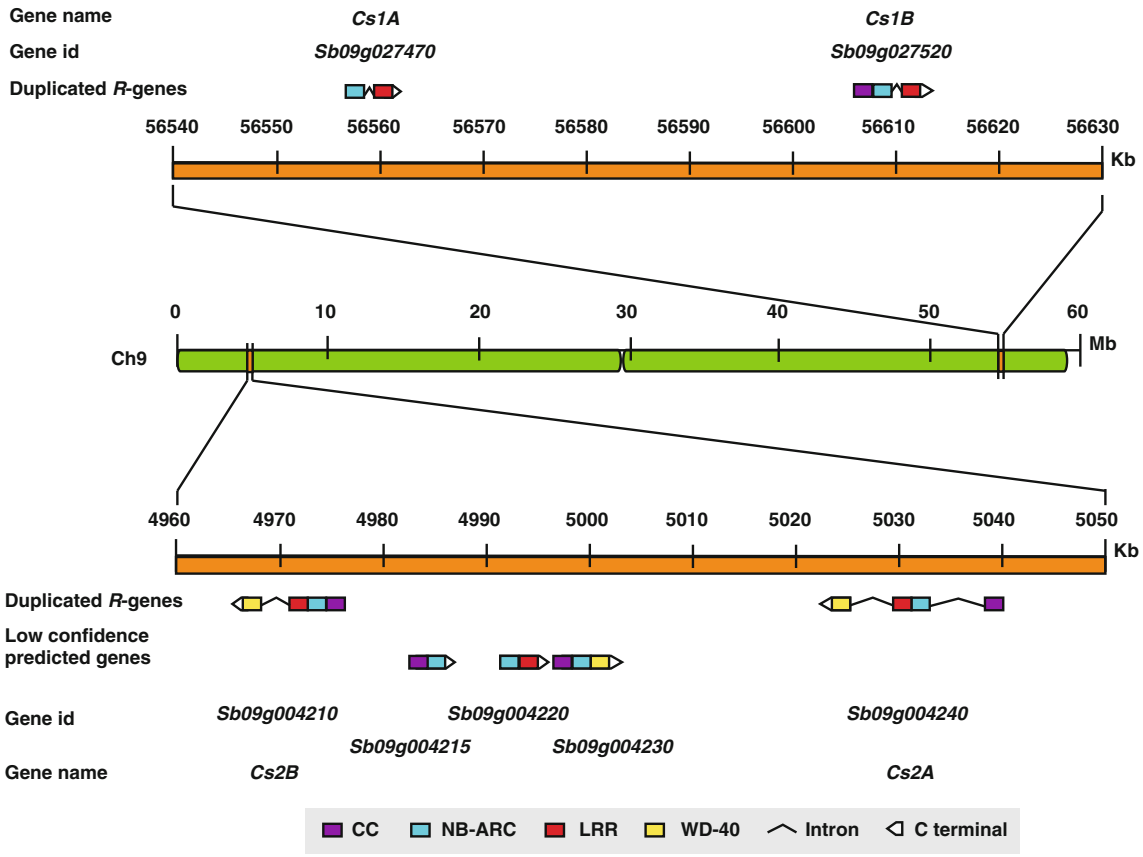

Fig. 4 Genomic regions with *Cs1* and *Cs2* loci (*orange–red*) on sorghum chromosome 9 (*green*). Genes in both loci and corresponding protein domains are schematically drawn

found in rice and slightly more compared to *Arabidopsis* (Paterson et al. 2009). Chromosome 5 in the sequenced BTx623 sorghum genotype showed the highest abundance of *R*-genes, 62 compared to 30, on chromosome 9. Furthermore, NB-LRR genes are commonly found in clusters in plant genomes (Meyers et al. 2003) but some remain as single copy loci. This division may reflect different evolutionary modes (McDowell and Simon 2006). For example, the *Cs2* locus may be a product of sequence exchanges and duplication (the rapid evolving Type-I class) whereas the *Cs1* locus could belong to the more conserved Type II class with infrequent recombination (Kuang et al. 2008). More sequence data from a larger collection of accessions from the sorghum genus is needed to support this hypothesis. Gene duplications have led to that *R*-gene pairs are common in plant genomes. In some cases both genes in a pair are required to interact with a single effector gene product (Eitas and Dangl 2010). But examples where either gene in a pair can provide resistance to a fungal pathogen are also known (Dixon et al. 1996). Because our VIGS construct were not designed to target individual *Cs* genes due to high sequence similarities, we cannot rule out that both genes in the two gene pairs contribute to *C. sublineolum* defense.

The NB-LRR encoded *Cs2* genes are either combined with the more frequent occurring CC domain or with a WD40 motif. The WD40 domain is one of the most abundant domains in eukaryotic proteomes and is involved in a large variety of cellular processes (Xu and Min 2011). It may function as an important substrate receptor and play crucial roles in vast numbers of protein–protein interactions, not least with the SCF ubiquitin ligase multi-component complex, leading to protein recycling and possibly to cell death (Pashkova et al. 2010; Xu and Min 2011). Because the hypersensitive response is a common cell death reaction to pathogens among plants, it has been speculated that WD40 domains play crucial roles in HR signaling cascades. However, none has yet shown such a link to disease resistance even though WD40 domains are present in plant disease resistance proteins (Drader and Kleinhofs 2010), and also in pathogen effectors (Qutob et al. 2006). On the other hand, we cannot exclude that the Cs2 proteins may have taken advantage of the function WD40 proteins have as transcription factors in the regulation of biosynthesis of anthocyanins, proanthocyanidins or in jasmonate-mediated anthocyanin accumulation (Pang et al. 2009; Martens et al. 2010; Qi et al. 2011). How such possible

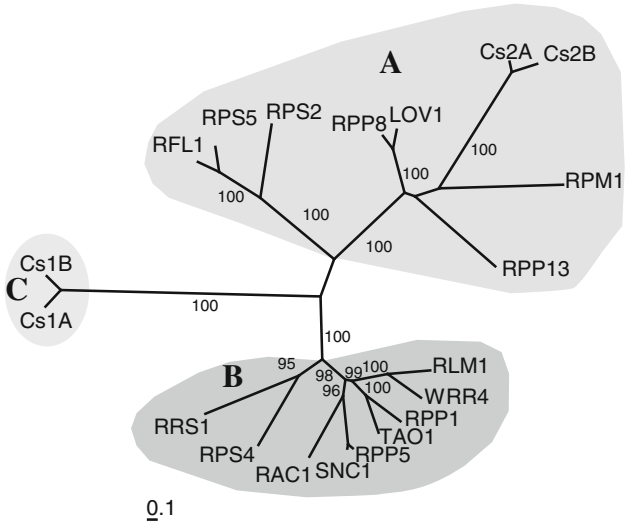

Fig. 5 Unrooted maximum-likelihood phylogram inferred from nucleotide binding (NB) and leucine rich repeat (LRR) domains, of four resistance proteins to *C. sublineolum* (Cs1A, Cs1B, Cs2A, Cs2B) in sorghum, compared with *Arabidopsis* NB-LRR resistance proteins with known function. LR-ELW values above 75% are shown. Labeling is as follows: **a** NB-LRR resistance proteins with NB-LRR proteins with a coiled-coil (CC) domain at the N-terminal end. **b** NB-LRR resistance proteins with a Toll/Interleukin-1 receptor (TIR) at the N-terminal end. **c** Two of the sorghum proteins not clustering with any *Arabidopsis* protein candidate. Units indicate substitutions/site. Accession numbers are listed in ESM Table S5

responses are triggered by *C. sublineolum* is far from understood as well as processes involving recognition of sorghum R-proteins. Much less is known about fungal effectors, which comprise diverse groups of molecules compared to the well-known Avr proteins from *P. syringae* (Mansfield 2009). No effectors from *C. sublineolum* are known today but the related *C. graminicola* genome (http://www.broadinstitute.org) may generate some valuable information on this line when sequencing is completed.

Interestingly, two TIR-NB-LRR encoding genes were found using a map-based cloning approach among *Arabidopsis* accessions to control resistance to a related pathogen, namely *Colletotrichum higginsianum*, denoted *RCH*. *C. higginsianum* causes anthracnose on cruciferious plants (Narusaka et al. 2009). The *RCH* genes were earlier identified as governing resistance to two bacterial pathogens, *RRS1* (resistance to *Ralstonia solanacearum 1*) and *RPS4* (resistance to *Pseudomonas syringae* pv. tomato—expressing *avrRps4*). Presently it is believed that different allelic forms of *RRS1* and *RPS4* collaboratively confer resistance to the different pathogens (Birker et al. 2009). *RRS1* and *RPS4* are distantly related based on sequence comparisons to the *Cs* genes found in this work (Fig. 5). Nevertheless, to elucidate any potential involvement of *RRS1* and *RPS4* in the defense to *C. sublineolum*, we inoculated *Arabidopsis* mutants impaired in those genes. No fungal colonization was observed on the mutants or on wild-type (Col-0, Ws-0) plants, suggesting that *RRS1* and *RPS4* are of minor or no importance for *C. sublineolum* in *Arabidopsis*.

A hallmark of R protein-mediated resistance is the hypersensitive response, often visible as a localized cell death response around the infection site. Interestingly, a cell death related protein was found to be of importance in our analysis. *SbCDL1* encodes a protein that shares 97.7% identity with the rice OsPCD5 protein. It was demonstrated that *OsPCD5* is important in the regulation of program cell death in rice from experiments where *OsPCD5* was over-expressed (Attia et al. 2005). However, any relationship with plant defense was not investigated. A direct link between the WD40 motif in Cs2 proteins and SbCDL1 is at this stage not possible to predict.

In addition to the earlier mentioned genes, we found four others that contribute to the *C. sublineolum* defense. They code for; a zinc finger-like transcription factor (SbZnTF1), a lipid transfer protein (SbLTP1), a casein kinase 2-like (SbCK2), and a defensin-like protein (SbDEFL1). All four of them, have homologous *Arabidopsis* genes that are reported to be associated with biotic stress responses. Furthermore, the up-regulated transcripts from genes in fatty acid metabolism and transport such as lipases, long-chain acyl-CoA synthetases and ceramidase found in the cDNA-AFLP analysis (ESM Table S2) may be of additional importance, particularly in priming the synthesis of plant defense signaling molecules (Kachroo and Kachroo 2009).

Taken together, the genes implicated in sorghum defense to *C. sublineolum* found in our study comprise *R*-genes, transcription factors and other defense-associated gene categories. They will form a basis for continued functional analysis of this particular plant host–pathogen interaction that is far from well understood. In parallel, the sequence information from the major contributors to the host defense responses, i.e., the *Cs1* and *Cs2* loci, will be converted into molecular markers and used in germplasm assessments and breeding programs in East Africa, an important development to sustain sorghum crop production in this part of the world.

Acknowledgments We greatly acknowledge B. Sarosh and S. Oide for assisting with the cDNA-AFLP work. This study was funded by the SIDA-SAREC grant for the East African Biotechnology Network (BIO-EARN), Sida SWE-2005-453, and the Swedish University of Agricultural Sciences.

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