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Research

A barley MLA immune receptor is activated by a fungal nonribosomal peptide effector for disease susceptibility

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Summary

- The barley *Mla* locus contains functionally diversified genes that encode intracellular nucleotide-binding leucine-rich repeat receptors (NLRs) and confer strain-specific immunity to biotrophic and hemibiotrophic fungal pathogens.
- In this study, we isolated a barley gene *Scs6*, which is an allelic variant of *Mla* genes but confers susceptibility to the isolate ND90Pr (Bs_{ND90Pr}) of the necrotrophic fungus *Bipolaris sorokiniana*. We generated *Scs6* transgenic barley lines and showed that *Scs6* is sufficient to confer susceptibility to Bs_{ND90Pr} in barley genotypes naturally lacking the receptor. The *Scs6*-encoded NLR (SCS6) is activated by a nonribosomal peptide (NRP) effector produced by Bs_{ND90Pr} to induce cell death in barley and *Nicotiana benthamiana*. Domain swaps between MLAs and SCS6 reveal that the SCS6 leucine-rich repeat domain is a specificity determinant for receptor activation by the NRP effector.
- *Scs6* is maintained in both wild and domesticated barley populations. Our phylogenetic analysis suggests that *Scs6* is a *Hordeum*-specific innovation.
- We infer that SCS6 is a *bona fide* immune receptor that is likely directly activated by the nonribosomal peptide effector of Bs_{ND90Pr} for disease susceptibility in barley. Our study provides a stepping stone for the future development of synthetic NLR receptors in crops that are less vulnerable to modification by necrotrophic pathogens.

Introduction

Plants have evolved an innate immune system that is constantly challenged by a wide variety of microbial pathogens with different lifestyles, each of which has evolved different strategies to manipulate the host and establish virulence. Interactions between plants and biotrophic pathogens, which must retrieve nutrients from living host cells to proliferate, are often subject to coevolution, with the pathogen restricted to a particular host species. The dynamics of these interactions are often driven by competing sets of co-evolving genes encoding plant immune receptors and pathogen effectors, the former being essential components for nonself-perception in the host and the latter being required for pathogen virulence (Saur *et al.*, 2021). Despite recent advances, our understanding of the evolutionary history and dynamics of plant interactions with necrotrophic pathogens that kill and feed on dying host cells is less understood, even though these pathogens cause substantial economic damage in crops (Newman & Derbyshire, 2020; Derbyshire & Raffaele, 2023a).

Necrotrophic pathogens may have a wide or narrow host range. The molecular basis of host generalism is not well defined, but appears to be linked to the repertoire of secreted cell wall-degrading enzymes (Newman & Derbyshire, 2020). Computational mining of pathogen genomes has revealed large arsenals of lineage- or species-specific effector proteins, often

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structurally related but with extreme divergence in their amino acid sequences (Sperschneider *et al.*, 2016; Derbyshire *et al.*, 2017; Lopez *et al.*, 2018; Le Marquer *et al.*, 2019; Seong & Krasileva, 2023; Derbyshire & Raffaele, 2023b). Experimental evidence shows that a subset of these effectors is required for virulence in necrotrophic pathogens with a narrow host range (Derbyshire & Raffaele, 2023a). Host-specialized necrotrophs often rely on proteinaceous or specialized metabolites that act as necrotrophic effectors (NEs) to induce host cell death and promote infection. *Pyrenophora tritici-repentis* produces the proteinaceous ToxA effector, which targets the extracellular C-terminal domain of the wheat transmembrane protein TaNHL10, but susceptibility depends on wheat *Tsn1*, which encodes an intracellular hybrid protein consisting of an N-terminal S/T protein kinase fused to an NLR composed of nucleotide-binding (NB) and leucine-rich repeat (LRR) domains (Faris *et al.*, 2010; Dagvadorj *et al.*, 2022). The necrotrophic pathogen *Parastagonospora nodorum* secretes the cysteine-rich proteinaceous effector SnTox1, which appears to directly target the plasma membrane-resident and wall-associated kinase (WAK) Snn1 for disease susceptibility in wheat (Liu *et al.*, 2009; Shi *et al.*, 2016).

Chemically diverse metabolites that act as NEs have been identified in the fungal genera *Cochliobolus*, *Corynespora*, and *Periconia*. The susceptibility of sorghum to *Periconia circinata* depends on the *Pc* locus, which encodes a cluster of three tandemly repeated *NLR* genes and production of chlorinated peptide toxins by the pathogen, called peritoxins (Dunkle & Macko, 1995; Nagy & Bennetzen, 2008). Loss of the central *NLR* results in loss of susceptibility to *P. circinata*, but it is unknown whether the toxin targets the NLR receptor directly or indirectly. The HC toxin of the causal agent of northern corn leaf spot, *Cochliobolus carbonum*, is a cyclic tetrapeptide and targets histone deacetylases of susceptible corn plants to establish infection (Brosch *et al.*, 1995; Walton, 2006). *Cochliobolus victoriae* deploys victorin toxin, a mixture of ribosomally encoded but highly modified hexapeptides, to induce cell death and establish infection on *Vb*-containing oat genotypes (Kessler *et al.*, 2020). *Vb* is genetically inseparable from *Pc-2*, which mediates disease resistance to the biotrophic pathogen *Puccinia coronata*, but it remains unclear whether they are the same or two closely linked genes (Mayama *et al.*, 1995; Wolpert & Lorang, 2016). Victorin toxin is sufficient to induce cell death in several 'nonhost' species, including *c.* 1% of accessions of *Arabidopsis thaliana* (Lorang *et al.*, 2004, 2007, 2010, 2012, 2018; Wolpert & Lorang, 2016). The NLR LOV1 in *A. thaliana* accession Cl-0 determines sensitivity to victorin, but also requires the thioredoxin *At*TRXh5, which contributes to salicylic acid-dependent defense through its denitrosylation activity on host proteins, including NPR1, the activator of systemic acquired resistance (Sweat & Wolpert, 2007; Tada *et al.*, 2008; Kneeshaw *et al.*, 2014). Victorin binds to *At*TRXh5 and inhibits its activity. Since *At*TRXh5 binds to LOV1 in the absence of victorin, it is proposed that the receptor senses the toxin indirectly through victorin-mediated perturbation of *At*TRXh5 activity (Lorang *et al.*, 2012).

Isolate-specific disease resistance to biotrophic or hemibiotrophic pathogens is often conferred by intracellular plant NLRs that directly or indirectly sense the presence of pathogen effectors. This results in receptor oligomerization and resistosome formation, inducing immune signaling and termination of pathogen proliferation. Canonical plant NLRs consist of three domains, a variable N-terminal signaling domain, a central nucleotide-binding oligomerization (NOD) domain, followed by a C-terminal leucine-rich repeat region (LRR) (Hu & Chai, 2023). Most plant NLRs carry either a Toll-interleukin-1 receptor-like (TIR) domain or a coiled-coil (CC) domain at the N-terminus and are referred to as TNLs and CNLs, respectively (Lapin *et al.*, 2022; Hu & Chai, 2023). The recognition specificity of sensor TNLs or CNLs is usually determined by their polymorphic LRR, whereas signaling NLRs become engaged in immune signaling initiated by sensor NLRs. CNL resistosomes integrate into host cell membranes and act as calcium-permeable channels that mediate Ca^{2+} influx, triggering immune signaling leading to host cell death (Wang *et al.*, 2019a,b; Bi *et al.*, 2021; Förderer *et al.*, 2022b). Sensor TNLs produce nucleotide-based second messengers that converge on the conserved EDS1 family to activate signaling/helper NLRs that carry a RESISTANCE TO POWDERY MILDEW 8 (RPW8)-CC domain (CC_R) (Wang *et al.*, 2019b; Ma *et al.*, 2020; Martin *et al.*, 2020; Jia *et al.*, 2022; Zhao *et al.*, 2022; S. Huang *et al.*, 2022; Förderer *et al.*, 2022a; Chai *et al.*, 2023). Similar to sensor CNL resistosomes, activated signaling NLRs of *A. thaliana* have calcium-permeable channel activity (Jacob *et al.*, 2021). Ca^{2+} influx and the accumulation of reactive oxygen species are key events in immune signaling and are tightly linked to a regulated death of host cells at sites of attempted pathogen ingress, the so-called hypersensitive response (HR) (Thordal-Christensen *et al.*, 1997; Grant *et al.*, 2000; Torres *et al.*, 2001). While the HR likely contributes to the termination of growth of biotrophic pathogens, it may promote the virulence of necrotrophs that retrieve nutrients from dying cells (Govrin & Levine, 2000).

Bipolaris sorokiniana (*Bs*) (teleomorph *Cochliobolus sativus*) is a necrotrophic fungus causing a wide range of diseases in cereals, including leaf spot blotch, common root rot, seedling blight and kernel blight (Kumar *et al.*, 2002). Although *Bs* can infect a wide range of grass species, strain-specific variation in virulence among a world-wide collection of isolates has been identified based on differential infection responses on a panel of barley accessions, distinguishing four *Bs* pathotypes (Valjavec-Gratian & Steffenson, 1997b; Zhong & Steffenson, 2001; Leng *et al.*, 2016). Major genes or QTLs for spot blotch resistance/susceptibility have been identified in various barley genotypes depending on the *Bs* pathotype (Valjavec-Gratian & Steffenson, 1997a; Bilgic *et al.*, 2005, 2006; Bovill *et al.*, 2010; Roy *et al.*, 2010; Grewal *et al.*, 2012; Wang *et al.*, 2017), but the dominant/recessive nature of each gene or QTL has yet to be determined in most cases. Recently, two wall-associated kinase genes, *Sbs1* and *Sbs2*, were isolated at the *Rcs5* locus, which confer susceptibility to spot blotch induced by the *Bs* isolate ND85F (Ameen *et al.*, 2020). Barley cultivar Bowman initially displayed moderate resistance to spot blotch when it

was released in North Dakota, USA, in 1985 (Franckowiak *et al.*, 1985). Only six years later, Bowman and cultivars derived from Bowman showed hyper-susceptibility to a newly emerged isolate of spot blotch, named Bs_{ND90Pr} (Fetch & Steffenson, 1994). This isolate belongs to *Bs* pathotype 2 and its high virulence on Bowman depends on the unique *VHv1* locus, which harbors a cluster of genes including two encoding nonribosomal peptide synthetases (NRPSs) (Valjavec-Gratian & Steffenson, 1997a; Zhong *et al.*, 2002; Condon *et al.*, 2013). Deletion of one of the two *NRPS* genes, termed *NPS1* (*ID#115356*), is sufficient to abolish the high virulence of Bs_{ND90Pr} on cultivar Bowman (Condon *et al.*, 2013). We recently identified *Scs6* as the dominant gene needed for susceptibility to spot blotch caused by Bs_{ND90Pr} in Bowman and physically anchored the locus to a 125 kb genomic region overlapping with the *Mla* locus in the barley cv. Morex reference genome (Leng *et al.*, 2018). Interestingly, the complex *Mla* locus is known to confer isolate-specific disease resistance to several foliar biotrophic pathogens, including the barley powdery mildew *Blumeria graminis* f. sp. *hordei* (*Bgh*), the stripe rust pathogen *Puccinia striiformis* and the hemibiotrophic blast pathogen *Magnaporthe oryzae* (Jørgensen & Wolfe, 1994; Seeholzer *et al.*, 2010; Maekawa *et al.*, 2019; Bettgenhaeuser *et al.*, 2021; Brabham *et al.*, 2023). The *Mla* locus harbors three NLR families, *Rgh1*, *Rgh2* and *Rgh3*, all of which encode CNL receptors (Wei *et al.*, 2002). For several MLA CNL immune receptors belonging to the RGH1 family, cognate pathogen effector proteins, termed avirulence effectors, have been isolated and at least some bind directly to the corresponding receptor (Lu *et al.*, 2016; Chen *et al.*, 2017; Saur *et al.*, 2019; Bauer *et al.*, 2021). Barley MLA immune receptors identified to date all belong to one of two MLA subfamilies from the RGH1 superfamily (Maekawa *et al.*, 2019).

Here, we used chemical mutagenesis of the susceptible cultivar Bowman to identify several Bs_{ND90Pr} resistant mutants. A customized Mutant Chromosome Sequencing (MutChromSeq) (Steuernagel *et al.*, 2017) approach was then used to identify independent mutations in the susceptibility factor *Scs6*, which we show to be a naturally occurring *Mla* allele present in 16% of domesticated barley germplasm. We generated *Scs6* transgenic barley in accessions lacking the receptor to show that *Scs6* is sufficient to confer Bs_{ND90Pr} susceptibility. We collected intercellular washing fluids (IWFs) from Bowman leaves inoculated with wild-type (WT) Bs_{ND90Pr} or the Bs_{ND90Pr} Δ*nps1* mutant and show that the former IWF is necessary and sufficient to reconstitute a cell death response in *Scs6*-containing barley and in *N. benthamiana* transiently expressing *Scs6*. Domain swaps between the SCS6 CNL and the MLA1 or MLA6 barley powdery mildew immune receptors and expression of the resulting hybrid proteins in *N. benthamiana* revealed that the SCS6 LRR domain determines sensitivity to the NPS1-derived effector. We performed Bs_{ND90Pr} inoculation experiments with a collection of wild barley lines to show that *Scs6* is maintained in multiple geographically separated wild barley populations. Phylogenetic analysis suggests that *Scs6* is a *Hordeum*-specific innovation. We infer that SCS6 is a *bona fide* immune receptor that is likely directly activated for disease susceptibility by the NPS1-derived effector of Bs_{ND90Pr}.

Materials and Methods

Plant materials and generation of EMS mutant population

The barley (*Hordeum vulgare* L.) cv. Bowman carrying *Scs6* (Leng *et al.*, 2018) was used to generate mutant lines that were resistant to spot blotch caused by *B. sorokiniana* isolate ND90Pr. The mutagenesis procedure was performed according to (Williams *et al.*, 1992) with some modifications (Fig. 1a). Approximately 2000 seeds of barley cv. Bowman were presoaked in 300 ml of phosphate buffer (0.05 M, pH 8.0) for 8 h at room temperature with gentle agitation. Then, the seeds were treated in 0.3% (v/v) Ethyl methanesulfonate (EMS) in phosphate buffer for 16 h at room temperature. Treated seeds were rinsed with water for 1 min and sown in pots immediately. The M_1 plants were grown in the greenhouse at 20 to 24°C under supplemental fluorescent lighting with a 16 h : 8 h, day : night cycle. Spikes were harvested separately from individual M_1 plants. Approximately 20 M_2 seedlings from each M_1 plant were screened for spot blotch resistance using isolate ND90Pr following the procedures and 1–9 disease rating scale described by (Fetch & Steffenson, 1999). Plants with an average disease score of 1 to 3 were classified as resistant, 4 to 5 as moderately resistant, and 6 to 9 as susceptible. Bowman was included as a positive control for susceptibility and ND5883 and NDB112 as positive controls for resistance. Resistant M_2 seedlings were selected and propagated by selfing to develop homozygous M_3 mutant lines, which were further confirmed for resistance to ND90Pr and then used for MutChromSeq analysis. Cultivated barley accessions from the USDA National Small Grains Collection (Leng *et al.*, 2016) and the Wild Barley Diversity Collection (WBDC) accessions (Roy *et al.*, 2010) were also screened against isolate ND90Pr using the same method as described above for the EMS mutants and used in the *Scs6* gene diversity study.

Fungal isolates, spot blotch phenotyping, Intercellular Washing Fluid (IWF) extraction and relative fungal biomass quantification

The pathotype 2 isolate ND90Pr of *B. sorokiniana* and the *NPS1* (ID# 115356) knockout mutants (*Δnps1* KO#3 and KO#9) were used for phenotyping throughout this research. Δ*nps1* KO#3 was previously described as Δ115356 (Condon *et al.*, 2013) and Δ*nps1* KO#9 was independently generated and isolated using the same method described by Condon *et al.* (2013). The disrupted gene structure in *Δnps1* KO#3 and KO#9 is shown in Supporting Information Fig. S1(c). V8 PDA (150 ml of V8 juice, 850 of ml H_2O, 10 g of PDA, 10 g of agar and 3 g of $CaCO_3$) was used to culture Bs_{ND90Pr} and fungal mutants under the conditions of 14 h of light and 10 h of darkness. Spore suspension containing 2×10^3 conidia ml^{-1} was prepared and sprayed on seedlings with the second leaves fully expanded (12–14 d after planting). Inoculated plants were incubated in a humidity chamber for 18–24 h and then transferred into the same greenhouse room. Disease ratings were conducted at 7 d postinoculation using the 1–9 rating scale of (Fetch & Steffenson, 1999).

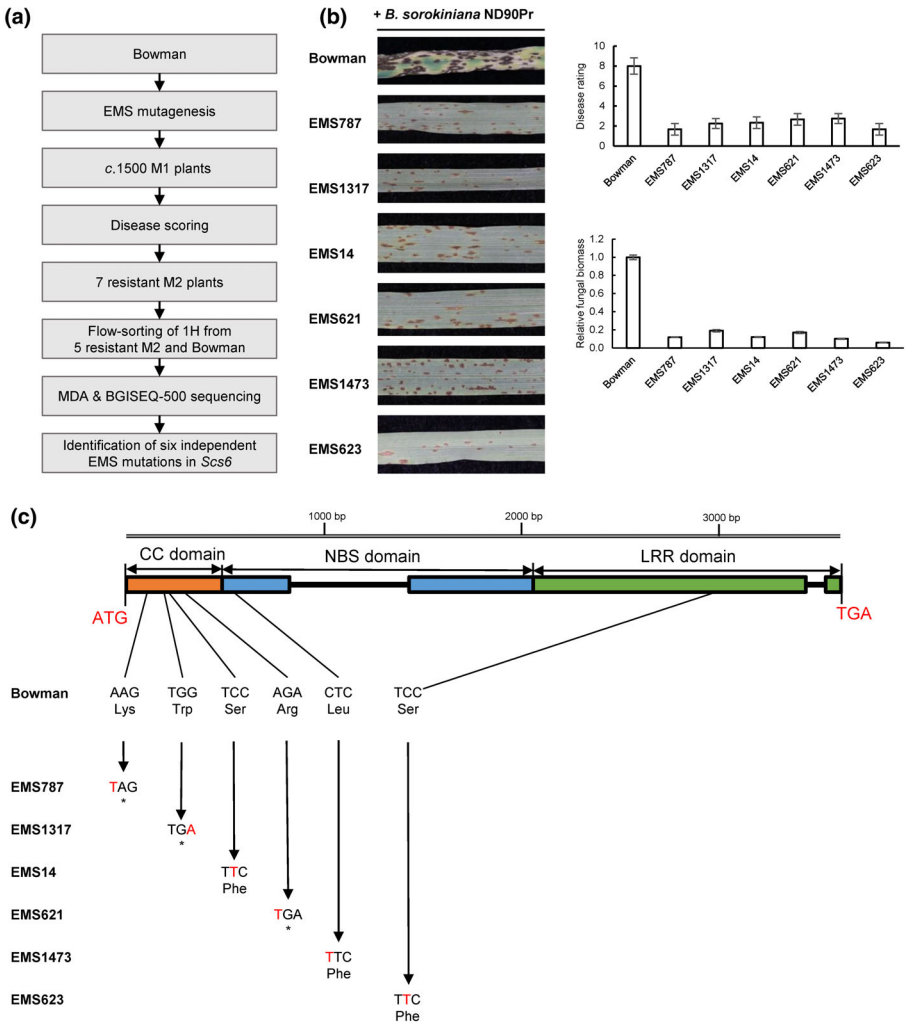

Fig. 1 Identification of *Scs6* by MutChromSeq. (a) Workflow for MutChromSeq. (b) Infection responses, disease scorings, and quantification of fungal biomass in Bowman and six barley Ethyl methanesulfonate (EMS)-induced M3 lines after inoculation with *Bipolaris sorokiniana* ND90Pr. Photos were taken at 7 d after inoculation. The 1–9 rating scale of Fetch & Steffenson (1999) was used to rate the spot blotch disease. Fungal biomass was quantified for Bowman and the six EMS-induced M3 lines using quantitative real-time polymerase chain reaction. The error bar indicates the SD. (c) Gene structure and EMS-induced mutations in *Scs6*, a gene encoding a canonical coiled-coiled-type NLR (CNL). * indicates stop codon. See also Supporting Information Fig. S3 and Tables S2–S3.

To prepare the IWF, barley cv. Bowman was inoculated with Bs_{ND90Pr} or Bs_{ND90Pr} Δ*nps1* KO#3 or Δ*nps1* KO#9 as described above, and leaves were harvested 7 d after inoculation. Harvested leaves were cut into fragments of *c.* 1 inch in length, and leaf fragments were submerged into distilled water in the beaker. The beaker was then set into a vacuum chamber and vacuumed for 30 min. Then, leaf fragments were surface-dried and transferred into 50-ml centrifuge tubes, which were centrifuged at 3200 ***g*** for 30 min. Finally, IWFs were harvested from the bottom of each centrifuge tubes, confirmed on barley cv. Bowman seedlings by infiltration, and stored at −20°C for further use.

To quantify the fungal biomass, DNA was extracted from the leaves harvested at 7 d after pathogen inoculation using a DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA). Subsequently, 50 ng of each DNA sample were used for quantitative real-time PCR (qPCR), which was performed using the ITS region as fungal target (ITS4 and ITS5, Table S1) and the actin gene of barley as reference (Actin-RT-F1 and Actin-RT-R1, Table S1). Real-time PCR was performed as described by (Condon *et al.*, 2013). The ITS CT values were normalized using the barley actin gene, and the relative gene copy number of ITS was calculated according to the $2^{-\Delta\Delta CT}$ method (Kumar *et al.*, 2015). The relative quantity of fungal biomass was calculated using barley cv. Bowman leaves inoculated with WT isolate ND90Pr as a control.

Flow sorting of barley chromosomes and preparation of DNA for sequencing

Suspensions of mitotic metaphase chromosomes were prepared from root tips of barley cv. Bowman carrying SCS6 and its five EMS mutants following (Lysák *et al.*, 1999). Briefly, root-tip cells were synchronized using hydroxyurea, accumulated in metaphase using amiprohos-methyl and fixed by formaldehyde. Intact chromosomes were released by mechanical homogenization of 100 root tips in 600 µl ice-cold LB01 buffer (Doležel *et al.*, 1989). GAA microsatellites on the isolated chromosomes were labelled by fluorescence *in situ* hybridization in suspension (FISHIS) using 5′-FITC-GAA7-FITC-3′ oligonucleotides (Sigma) according to (Giorgi *et al.*, 2013) and chromosomal DNA was stained by DAPI (4′,6-diamidino 2-phenylindole) at 2 µg ml^{-1}. Bivariate chromosome analysis and sorting was done using a FACSAria II SORP flow cytometer and sorter (Becton Dickinson Immunocytometry Systems, San José, CA, USA). Sort window delimiting the population of chromosome 1H was setup on a dot-plot FITC-A vs

DAPI-A and 55 000–70 000 copies of 1H chromosomes were sorted from each sample at rates of 1500–2000 particles per second into PCR tubes containing 40 µl sterile deionized water. Chromosome content of flow-sorted fractions was checked by microscopic observation of 1500–2000 chromosomes flow sorted into 10 µl drop of PRINS buffer containing 2.5% sucrose (Kubaláková *et al.*, 1997) on a microscopic slide. Air-dried chromosomes were labelled by FISH with a probe for GAA microsatellite according to (Szakács *et al.*, 2013). In order to determine chromosome content and the purity, which was expressed as percent of 1H in the sorted fraction, at least 100 chromosomes in each sorted sample were classified following the molecular karyotype of barley (Szakács *et al.*, 2013). The samples of flow-sorted chromosomes 1H were treated with proteinase K, after which their DNA was amplified by multiple displacement amplification (MDA) (Table S2) using an Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Chalfont St. Giles, UK) as described by (Šimková *et al.*, 2008). The DNA samples were sequenced by BGI using BGISEQ-500 (Cambridge, MA, USA) to generate 100-bp paired-end (PE) reads.

MutChromSeq

Raw sequencing data from flow-sorted chromosome 1H of the WT and EMS mutants were quality-trimmed using TRIMMOMATIC (Bolger *et al.*, 2014). The Bowman 1H chromosome sequencing data was assembled using ABySS 2.0 (Simpson *et al.*, 2009; Jackman *et al.*, 2017) and was masked for repeats using REPEATMASKER (http://repeatmasker.org). Sequence reads from EMS mutants were aligned to the repeats-masked Bowman 1H assembly using software BWA (Li & Durbin, 2009). The reads-aligned bam files were further processed using SAMTOOLS 0.1.19 (Li & Durbin, 2009) following parameters suggested by (Steuernagel *et al.*, 2016). The resulting pileup formatted files for WT and EMS mutants were used as the inputs analyzed by Pileup2XML.jar (https://github.com/steuernb/MutChromSeq). Finally, MutChromSeq.jar (https://github.com/steuernb/MutChromSeq) was executed to identify the candidate contigs with mutations in EMS mutants analyzed. All mutations were manually validated using INTEGRATIVE GENOMICS VIEWER software (IGV, v.2.5.2, Robinson *et al.*, 2011).

Identification of the candidate gene for *Scs6* and quantification of the relative expression of *Scs6* by quantitative real-time PCR

Gene annotation for the MutChromSeq-identified contig with mutations in EMS mutants was performed by FGENESH (Solovyev *et al.*, 2006). The genomic structure of *Scs6* was confirmed by PCR sequencing using both genomic DNA and cDNA as templates and primers listed in Table S1. *Scs6* was amplified by PCR from the five EMS mutants used in MutChromSeq and three additional EMS mutants with primer pair SCS6-F2/SCS6-R17 (Table S1).

To analyze the relative expression of *Scs6*, quantitative reverse transcription polymerase chain reaction was performed. The total RNA was extracted from barley leaves at nine time points (0, 6, 12, 24, 36, 48, 72, 96 and 120 h) after pathogen inoculation using the Total RNA Miniprep Kit (NEB, Ipswich, MA, USA). 2 µg of total RNA was used in the reverse transcription PCR to synthesize complementary DNA (cDNA) using ultrapure SMART MMLV reverse transcriptase (Takara Bio, San Jose, CA, USA) according to the manufacturer's protocol. cDNA was diluted 20 times and used as the template for quantitative reverse transcription polymerase chain reaction, which was performed with the CFX96 real-time PCR system (Bio-Rad). Real-time polymerase chain reaction was performed as described by Condon *et al.* (2013). All samples were normalized using the internal reference gene of Actin (Actin-RT-F1 and Actin-RT-R1, Table S1) and the relative expression of *Scs6* was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) and the sample collected from 0 h after inoculation was used as the control.

Binary vector construction and *Agrobacterium*-mediated transformation of barley

To determine the function of *Scs6*, two binary vectors for expression of *Scs6* (Fig. S2) were constructed and used to transform Golden Promise and SxGP DH-47 (DH47), which are resistant to isolate ND90Pr, using the *Agrobacterium*-mediated transformation method. The whole coding sequence (CDS) of *Scs6* was synthesized by GenScript (Piscataway, NJ, USA) and inserted between the *Spe*I and *Bsr*GI restriction sites of the binary vector pANIC12A (Mann *et al.*, 2012), producing a new construct pANIC12A-*Scs6* with the *Scs6* gene driven by a *Ubi* promoter and stopped by a *NOS* terminator. Another binary vector based on pBract202 (Smedley & Harwood, 2015) was constructed (pBract202-pMla6-*Scs6*-tMla6, Fig. S2), which carries the coding sequence of candidate *Scs6* flanked by the 5′ and 3′ regulatory sequences of *Mla6*. The two binary vectors pANIC12A-*Scs6* and pBract202-pMla6-*Scs6*-tMla6 were introduced into barley cv. Golden Promise and DH47 by *Agrobacterium*-mediated transformation following the methods described by Bartlett *et al.* (2008) and Brabham *et al.* (2023), respectively.

Transient gene expression in *N. benthamiana* and protein detection by immunoblotting

Generation of entry and destination vectors for expression of MLA1, MLA6, MLA22, and AVR_{A1} and AVR_{A6} is described in Saur *et al.* (2019), Bauer *et al.* (2021). The WT coding sequence without the stop codon of *Scs6* and of *MLA16*, *MLA18*-1 and *MLA25* (Seeholzer *et al.*, 2010) was amplified by PCR using attB-primers followed by BP reaction into pDONR221 to generate a gateway-compatible entry clone (Table S1). Entry vectors carrying WT cDNAs of *MLA3*, *FT153*, *FT352-2* and *MLA18-2* without stop codons and insect-cell codon-optimized *ScSr50* were obtained by gene synthesis (GeneArt; Invitrogen). Plasmids encoding chimeric SCS6/MLA1 and SCS6/MLA6 receptors were assembled using the NEBuilder HiFi assembly Kit (NEB) based on the domain boundaries reported in (Shen *et al.*, 2003). pENTR221-*Scs6* was used as a template to generate $Scs6^{S793F}$

and *Scs6*^{H502V} via PCR mutagenesis using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA).

LR-Clonase II (Thermo Fisher Scientific, Waltham, MA, USA) was used to recombine the genes into the expression vector pGWB517 that carries a C-terminal linker region followed by an in-frame 4xmyc epitope tag (Nakagawa *et al.*, 2007). The integrity of all entry and destination vectors was confirmed by whole-plasmid nanopore sequencing (Eurofins Genomics, Louisville, KY, USA). Expression constructs were transformed into *Agrobacterium tumefaciens* GV3101 (pMP90RK) by electroporation. Transformants were selected for 3 d at 28°C on LB agar medium containing rifampicin (15 mg ml^{-1}), gentamycin (25 mg ml^{-1}), kanamycin (50 mg ml^{-1}), and spectinomycin (50 mg ml^{-1}). Transformants were cultured in liquid LB medium containing the corresponding antibiotics at 28 h overnight, after which they were harvested by centrifugation at 2500 ***g*** for 6 min and resuspended in infiltration buffer (10 mM MES pH 5.6, 10 mM $MgCl_2$, and 200 μM acetosyringone). Transient gene expression in leaves of 4-wk-old *N. benthamiana* plants was performed via Agrobacterium-mediated transient expression assays in the presence of the P19 and CMV2b suppressors of RNAi silencing (Norkunas *et al.*, 2018). The final OD_{600} of bacteria carrying expression vectors of immune receptors and silencing suppressors was set to 0.5, unless stated otherwise. For the expression of effector proteins, the OD_{600} was increased to 1.0 unless stated differently. Twenty-four hours after *agrobacterium*-mediated gene delivery, IWF was infiltrated, as indicated. For this, a small subset toward the outer part of the region of transgene expression was infiltrated with *c.* 25–50 μl of IWF. Cell death phenotypes were assessed and documented at 2 or 4 d after agroinfiltration for IWF-triggered cell death or AVR_A-triggered cell death, respectively.

Ion leakage assays were performed as described in Lapin *et al.* (2019). After agroinfiltration into *N. benthamiana*, the plants were placed under a 16 h : 8 h, light : dark growth chamber at 23°C. 6-mm leaf discs from *N. benthamiana* agroinfiltrated leaves were collected at 24 h after agroinfiltration. IWF was infiltrated before that at 18 h after agroinfiltration, as indicated. The leaf discs were washed in 15 ml of Milli-Q water (5 MΩ*cm) for 30 min, and subsequently transferred to a 48-well plate with 0.5 ml Milli-Q water in each well, and incubated in a growth chamber with constant light. Ion leakage was measured at 6 h after with a Horiba Twin Model B-173 conductometer. For statistical analysis, results of measurements of 6 individual leaf discs were combined from 3 experiments. One-way ANOVA analysis was used and significantly different values were labelled with different letters (adjusted P value < 0.05).

For the detection of protein accumulation, leaf material of four individual plants was harvested 48 h after infiltration, flash-frozen in liquid nitrogen and ground to powder using a Retsch bead beater. Then, 100 mg plant tissue powder was resuspended in 200 μl Urea-SDS sample buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 8 M Urea, 4% β-mercaptoethanol, 5% Glycerol and 0.004% bromophenol blue) and vortexed at room temperature for 10 min. After centrifugation at 16 000 ***g*** for 15 min, 10 μl of supernatant were loaded onto a 10% SDS-PAGE without prior boiling. Separated proteins were transferred to a PVDF membrane and probed with monoclonal mouse anti-Myc (1 : 3000, R950-25; Thermo Fisher Scientific), polyclonal rabbit anti-GFP (1 : 3000, pabg1; Chromotek, Planegg, Germany) followed by polyclonal goat anti-mouse IgG-HRP (1 : 7500, ab6728; Abcam, Cambridge, UK) or polyclonal swine anti-rabbit IgG-HRP (1 : 5000, PO399; Agilent DAKO, Santa Clara, CA, USA) antibodies. Myc-tagged proteins were detected using SuperSignal West Femto: SuperSignal substrates (Thermo Fisher Scientific) in a 1 : 1 ratio. SuperSignal Femto Substrate was used for AVR_{A1} and SuperSignal Substrate for AVR_{A6}.

Sequencing of *Scs6* homologs in cultivated and wild barley accessions

The primer pair SCS6-F2 and SCS6-R17 (Table S1) was used to amplify the whole gene of *Scs6* in cultivated and wild barley accessions (Datasets S1, S2). PCR products were purified using Quick PCR Purification Kit (Thermo Fisher Scientific) and sequenced by EurofinGenomics (Louisville, KY, USA) using primers F2, R2, R3, SCS6-Seq-R1, SCS6-Seq-F1, and SCS6-Seq-F2 (Table S1). Homologs were aligned against the CDS of *Scs6* and any single nucleotide polymorphism (SNP) was validated by checking the sequence quality manually. Finally, the sequences of *Scs6* homologs excluding introns were translated into amino acid sequences and used for phylogenetic analysis.

Phylogenetic analysis of *Scs6* and *Mla* alleles

Previously published MLA protein sequences were retrieved from NCBI and aligned via SnapGene using Clustal Omega. Protein sequences of SCS6 variants in wild barley identified in this study were manually added to the alignment (Datasets S3, S4). A BLAST search was conducted to identify MLA-like sequences in the Triticeae using MLA1 and SCS6 as an input. The identified candidate sequences were manually inspected to remove truncated (> 840 aa) sequences. The resulting alignment was used to generate neighbor-net networks as described in Maekawa *et al.* (2019) using splitstree4 (Huson & Bryant, 2006). We regarded the first N-terminal 161 amino acids that align with SCS6 as the CC domain, the sequence stretching from amino acid 162 to 551 as the NB domain, and the sequence from amino acid 551 to the end as the LRR. To analyze sites undergoing positive selection, the Clustal alignment of protein sequences as well as the corresponding nucleotide coding sequences were used as an input for PAL2NAL to generate a codon-aware MSA (Dataset S5). In this MSA, sites under episodic positive selection were identified using the MEME algorithm (Murrell *et al.*, 2012) with default parameters and sites under pervasive positive selection identified using FUBAR (Murrell *et al.*, 2013) with default settings. Both MEME as well as FUBAR were accessed via the datamonkey application (Weaver *et al.*, 2018). The maximum-likelihood tree was constructed using MEGA11 using the bootsstrap method (100 replications) and based on amino acid substitutions with the Jones-Taylor-Thornthon (JTT) method.

Geographic distribution of wild barley accessions susceptible to Bs_{ND90Pr}

The geographic coordinates of sampled accessions from the WBDC (Roy *et al.*, 2010) and (Maekawa *et al.*, 2019) were plotted in QGIS 3.32. Geographic vector map datasets were downloaded from the Natural Earth repository (http://www.naturalearthdata.com).

Results

SCS6 is a naturally occurring variant of MLA subfamily 2 CNL receptors

To molecularly isolate *Scs6*, we applied the MutChromSeq approach (Steuernagel *et al.*, 2017) (Fig. 1a). We first mutagenized seeds of the susceptible barley cultivar Bowman with ethyl methanesulfonate (EMS; Williams *et al.*, 1992) and screened M_2 families derived from *c.* 1500 M_1 plants by inoculation of the seedlings with *B. sorokiniana* isolate ND90Pr (Bs_{ND90Pr}) (Fig. 1a; Materials and Methods section). A total of seven resistant M_2 families (EMS14, EMS494, EMS621, EMS623, EMS787, EMS1317 and EMS1473) were identified, each characterized by drastically reduced cell death lesion formation in Bs_{ND90Pr}-inoculated leaves compared to WT Bowman (Fig. 1b). Next, we flow-sorted chromosome 1H from five of the resistant EMS mutants and WT Bowman (Fig. S3), performed multiple displacement amplification (MDA) and BGISEQ-500 DNA sequencing of the 1H chromosomes (Table S2). We mapped sequence reads of each mutant line to the Bowman 1H assembly using the MutChromSeq pipeline and identified only one Bowman scaffold (scaffold_4918245 with a length = 23 130 bp) that was mutated in four mutant lines (EMS14, EMS621, EMS1317 and EMS1473) or deleted (the whole 23 130 bp sequence was missing) in one mutant (EMS494) (Table S3). The four mutant lines (EMS14, EMS621, EMS1317 and EMS1473) each carry different nonsynonymous single nucleotide substitutions in a single gene (Fig. 1c). These substitutions are consistent with EMS alkylating activity on guanine residues and result in either premature stop codons or deduced single amino acid substitutions in the 5′ coding region of a candidate *Scs6* gene (Fig. 1c). Targeted genomic DNA resequencing of this gene, amplified by PCR from all seven mutant lines, validated the MutChromSeq analysis and identified two additional EMS mutant lines, EMS787 and EMS623, each carrying unique nonsynonymous single nucleotide substitutions that resulted in a premature stop codon in the 5′ or a deduced single amino acid substitution in the 3′ coding region, respectively, making it likely that the corresponding WT gene is *Scs6* (Fig. 1c). The deduced protein of candidate *Scs6* consists of 959 amino acids with a tripartite domain organization typical of canonical CNL-type immune receptors, that is an N-terminal coiled-coil domain (CC), a central nucleotide-binding domain (NB), and C-terminal leucine-rich repeats (LRRs) (Fig. 1c). Protein sequence alignment with MLA/RGH1 variants found in multiple wild barley populations identified the candidate SCS6 as a novel member of the MLA receptor subfamily 2 (Maekawa *et al.*, 2019). This subfamily differs from MLA subfamily 1 mainly by polymorphisms in the CC domain, but both subfamilies have an overall high protein sequence similarity of at least 88%.

Scs6 is necessary and sufficient to confer susceptibility to Bs_{ND90Pr} in barley

To further confirm that the candidate *Scs6* confers susceptibility to Bs_{ND90Pr} in barley, we generated transgenic plant lines in Bs_{ND90Pr}-resistant barley cultivar Golden Promise (GP) and barley line SxGP DH-47 (DH47) using two binary vectors that carry the coding sequence of candidate *Scs6* flanked either by the maize *Ubi* promotor and *NOS* terminator sequences or by 5′ and 3′ regulatory sequences of barley *Mla6*, respectively (Fig. S2). The *Scs6* transgenic plants obtained from both GP and DH-47 genetic backgrounds showed a strong susceptibility reaction to Bs_{ND90Pr} when *Scs6* transgene copies were present (Figs 2, S4; Table S4), validating that the candidate gene is *Scs6*. We monitored the gene expression of *Scs6* in Bowman, EMS14 as well as in the transgenic *Scs6* line $DH47_{04659\text{-}8\text{-}4}$ upon pathogen challenge or without pathogen infection. We observed *Scs6* expression in both pathogen-inoculated

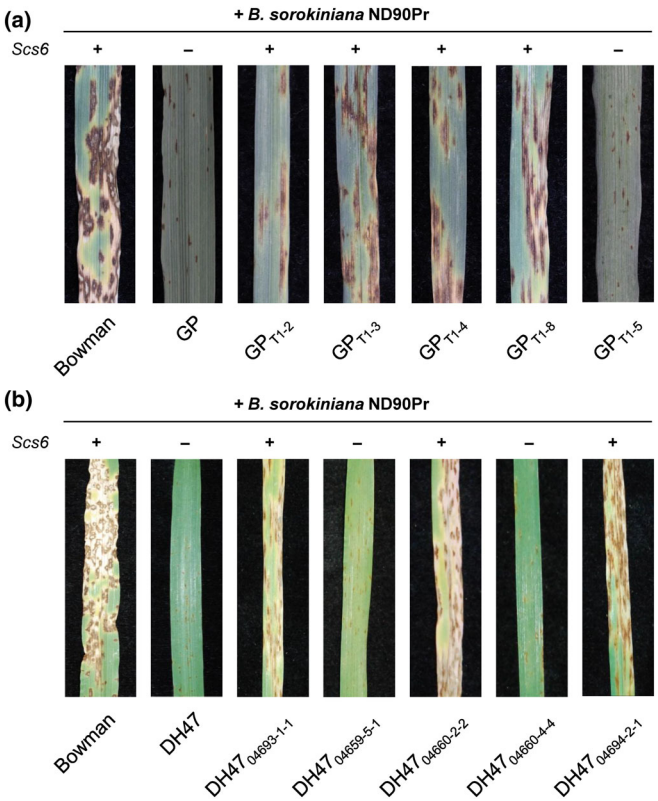

Fig. 2 *Scs6* is necessary and sufficient to confer susceptibility to *Bipolaris sorokiniana* ND90Pr in barley. (a) Representative images of infection responses of Golden Promise (GP) and derived transgenic *Scs6* T1 plants to *B. sorokiniana* ND90Pr, 7 d after inoculation. (b) Representative images of infection responses of SxGP DH47 (DH47) and derived transgenic *Scs6* T3 plants to *B. sorokiniana* ND90Pr, 7 d after inoculation. + indicates the presence of *Scs6*; – indicated the absence of *Scs6*. See also Supporting Information Figs S1, S2, S4, S5 and Table S4.

and noninoculated Bowman plants, indicating that *Scs6* expression is not induced by pathogen infection (Fig. S5). Expression profiles of *Scs6* were similar in Bowman and EMS14 after inoculation with Bs_{ND90Pr} or its NPS1 mutant (*Δnps1* KO#3), suggesting that *Scs6* expression is not impacted by mutations at *Scs6* or pathogen challenge regardless of the NPS1-derived effector (Fig. S5). However,

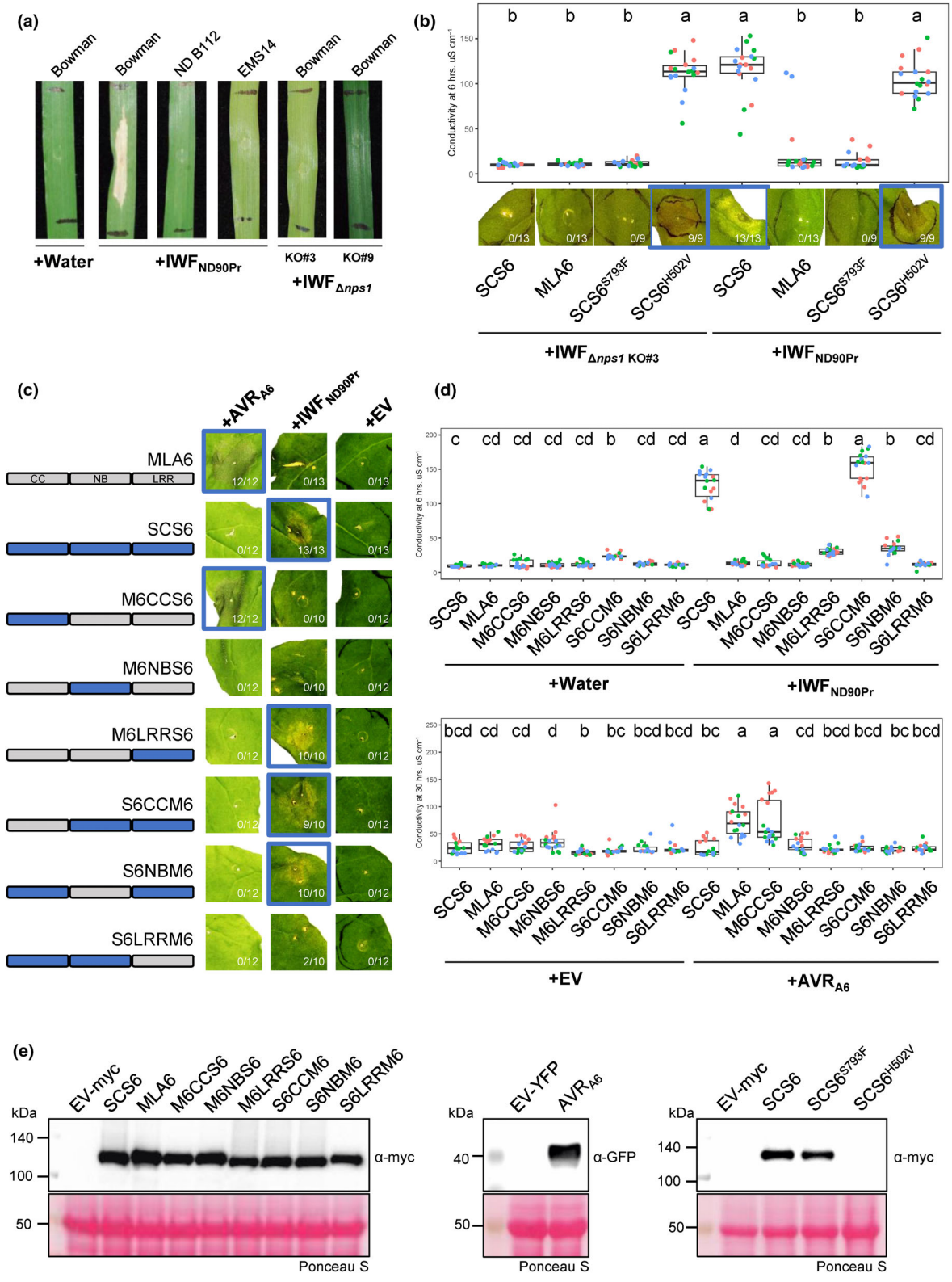

Fig. 3 *Bipolaris sorokiniana* ND90Pr secretes an effector that activates Scs6 via its LRR region to cause cell death in barley and *Nicotiana benthamiana*. (a) Barley genotypes that express *Scs6* (Bowman) or negative control (ND B112) were infiltrated with intercellular washing fluid (IWF) that was isolated from Bowman leaves infected either with wild-type (WT) *B. sorokiniana* ND90Pr (IWF_{ND90Pr}) or mutants *B. sorokiniana* Δ*nps1* KO#3 and KO#9 ($IWF_{\Delta nps1}$ KO#3 and KO#9), as indicated. (b, c) *Nicotiana benthamiana* plants were transformed transiently, as indicated. Genes were fused in between the 35S promotor sequence and 4xmyc (receptors) or mYFP (AVR_{A6} without signal peptide) epitope sequences. Twenty-four hours after *Agrobacterium*-mediated gene delivery, IWF_{ND90Pr}, $IWF_{\Delta nps1\ KO\#3}$ or water was infiltrated, as indicated. Cell death phenotypes were assessed and documented at 2 or 4 d after agroinfiltration for IWF-triggered cell death or AVR_A-triggered cell death, respectively. Representative pictures of at least six biological replicates (indicated in brackets) are shown and combinations that resulted in cell death are highlighted with a blue box. CC, Coiled-coil domain; EV, Empty vector; LRR, Leucine-rich repeat domain; NB, Nucleotide-binding domain. (d) Ion leakage assays of cell death in *N. benthamiana* leaves after agroinfiltration of the indicated constructs. Results from three independent biological experiments (n = 18, 6 leaf disks for each experiment). Each individual dot represents one measurement and the colors indicate the corresponding biological replicate. In each box, the top, middle and lower horizontal lines correspond to the upper quartile, median and lower quartile values, respectively. Whiskers depict the maximum and minimum values, dots above or below the whiskers are being considered outliers. Letters indicate significant differences (analyzed by one-way ANOVA with Tukey's multiple comparisons test, adjusted P value < 0.05). (e) For determination of protein levels of receptor-4xMyc (*c.* 114 kDa and AVR_{A6}-mYFP (39 kDa)) in *Nicotiana benthamiana*, leaf tissue was harvested 2 d post *Agrobacterium* infiltration. Western Blots are based on at least two replicates. See also Supporting Information Figs S6–S13.

the expression profile of *Scs6* in the transgenic *Scs6* line $DH47_{04659\text{-}8\text{-}4}$ was different from those in Bowman and EMS14 after pathogen inoculation (Fig. S5). This could be due to different genetic background (DH47) or promoter (*Mla6* promoter) used for driving the *Scs6* expression or a combination of the two factors. Taken together, we conclude that *Scs6* is not only necessary for susceptibility to Bs_{ND90Pr} in cultivar Bowman but also sufficient to confer susceptibility to the fungal pathogen when introduced as transgene in both tested resistant barleys lacking the receptor.

Barley SCS6 is activated by a Bs_{ND90Pr} nonribosomal peptide effector to induce cell death in barley and *N. benthamiana*

In previous studies, we identified two fungal genes in Bs_{ND90Pr} which encode a nonribosomal peptide synthetase (NRPS; *NPS1*) and a 4′-phosphopantetheinyl transferase (PPTase), respectively (Leng & Zhong, 2012; Condon *et al.*, 2013). Both NPS1 and PPTase are necessary for Bs_{ND90Pr} to become virulent and induce necrotic lesions in Bowman leaves, and PPTase is required for activation of the NRPS enzyme (Leng & Zhong, 2012; Condon *et al.*, 2013). We inoculated the barley lines Bowman, Golden Promise, DH47 as well as the *Scs6* transgenic lines $GP_{T1\text{-}3}$ and $DH47_{04659\text{-}8\text{-}4}$ with two independent *NPS1* mutants (*Δnps1* KO#3 and *Δnps1* KO#9). The results confirmed that *Scs6*-mediated susceptibility to Bs_{ND90Pr} depends on *NPS1* (Fig. S1). We hypothesized that Bs_{ND90Pr} synthesizes and delivers a nonribosomal peptide effector inside barley cells to induce SCS6-mediated cell death thereby facilitating its necrotrophic growth. We attempted to produce the effector by *in vitro* culture of Bs_{ND90Pr} in nutrient-limited media, but the fungal culture filtrates did not elicit necrotic symptoms after infiltration into Bowman leaves (data not shown). We reasoned that the fungus might produce the effector during infection *in planta*. Therefore, we inoculated Bowman seedlings with WT Bs_{ND90Pr} and collected Intercellular Washing Fluid (IWF) from leaves seven days after the inoculation (denoted IWF_{ND90Pr}). When IWF_{ND90Pr} was infiltrated into healthy leaves of susceptible Bowman, resistant ND B112, resistant EMS14 and previously characterized double-haploid (DH) progeny derived from a cross between susceptible Bowman and resistant Culicuchima (Leng *et al.*, 2018), only susceptible barley lines harboring *Scs6* developed necrotic lesions at the sites of IWF infiltration (Figs 3a, S6a,b). This indicates that susceptibility to isolate Bs_{ND90Pr} and cell death activity of IWF_{ND90Pr} both depend on the presence of *Scs6*. IWF collected from barley leaves inoculated with two independent *NPS1* knockout mutants (Bs_{ND90Pr} Δ*nps1* KO#3 and KO#9 with the corresponding IWF denoted as $IWF_{\Delta nps1}$ KO#3 and KO#9, respectively) failed to induce necrotic leaf lesions in *Scs6*-containing barley lines (Fig. 3a). Cell death activity of IWF_{ND90Pr} on Bowman was retained upon prolonged heat treatment of the IWF but lost after proteinase K incubation, consistent with an NRPS-derived effector (20 min 95°C; Fig. S6c). Collectively, these results suggest that Bs_{ND90Pr} secretes a nonribosomal peptide effector that can be recovered by IWF extraction, to trigger *Scs6*-dependent cell death in barley.

To investigate whether SCS6 can serve as a target of the Bs_{ND90Pr}-derived NRP effector, we expressed the barley CNL in leaves of heterologous *Nicotiana benthamiana*, a dicotyledonous plant. We delivered WT *Scs6* or a *scs6* mutant via *Agrobacterium tumefaciens* infiltration. *Scs6* expression in *N. benthamiana* caused a rapid and robust induction of cell death after infiltration of IWF_{ND90Pr} but not $IWF_{\Delta nps1}$ KO#3 (Fig. 3b). Expression of *scs6* present in EMS mutant 623 ($SCS6^{S793F}$) followed by IWF_{ND90Pr} infiltration failed to result in a cell death response. This is consistent with the finding that the EMS mutant 623 in barley is resistant to isolate Bs_{ND90Pr} (Fig. 1c), suggesting that the corresponding single amino acid substitution S793F in the SCS6 LRR domain renders the protein insensitive to the Bs_{ND90Pr}-derived effector (Fig. 3b). Expression of a *Scs6* variant ($SCS6^{H502V}$) resulting from a single amino acid substitution in the conserved MHD motif of the NB domain rendered SCS6 autoactive, that is $SCS6^{H502V}$-mediated cell death in *N. benthamiana* occurred in the absence of IWF_{ND90Pr} (Fig. 3b). Equivalent substitutions in the MHD motif have been shown to result in autoactive MLA immune receptors triggering cell death *in planta* in the absence of matching *Bgh* avirulence effector proteins (Bai *et al.*, 2012). WT SCS6 and $SCS6^{S793F}$ accumulated to similar steady-state levels in *N. benthamiana* leaf tissue (Fig. 3e). However, the autoactive $SCS6^{H502V}$ variant was undetectable,

presumably because little or no protein was produced due to immediate onset of cell death following *Agrobacterium*-mediated delivery of the corresponding gene construct (Fig. 3e). In *N. benthamiana* transiently expressing *Scs6*, IWF_{ND90Pr} treated at 95C for 5 min remained active in triggering the cell death, but lost its activity after the proteinase K treatment (Fig. S6d). Taken together, these results demonstrate that barley *Scs6* expression in heterologous *N. benthamiana* is sufficient to recapitulate an IWF_{ND90Pr}-dependent cell death.

Bs_{ND90Pr}-delivered effector specifically activates SCS6 via its LRR and NB domains

To further characterize SCS6-mediated cell death *in planta*, we constructed a series of hybrid receptors between SCS6 and MLA subfamily 1 immune receptors MLA6 or MLA1, guided by their shared modular domain architecture. The respective gene constructs were expressed in *N. benthamiana* following agroinfiltration and tested for their ability to induce cell death in the presence of matching *Bgh* avirulence effectors, AVR_{A1} or AVR_{A6}, or IWF_{ND90Pr} or $IWF_{\Delta nps1}$ (Figs 3, S7–S13; Bauer *et al.*, 2021). MLA1 and MLA6 were activated by cognate avirulence effectors AVR_{A1} and AVR_{A6}, respectively, but not IWF_{ND90Pr}, indicating that MLA recognition specificities for the proteinaceous and nonribosomal peptide effectors are retained despite receptor overexpression. Hybrid receptors constructed through the exchange of the N-terminal CC domain of MLA1 or MLA6 with the corresponding sequence-diverged CC domain of SCS6 retained the ability to detect *Bgh* effectors AVR_{A1} or AVR_{A6}, respectively (Figs 3, S7–S13). This is consistent with previous data showing that recognition specificities of MLA1 and MLA6 for the matching *Bgh* avirulence effectors are mainly determined by their polymorphic C-terminal LRRs (Shen *et al.*, 2003). Similarly, SCS6 hybrids carrying the CC domain of either MLA1 or MLA6 retained the ability for cell death activation upon IWF_{ND90Pr} infiltration (Figs 3, S7–S13). This indicates that the CC domains of SCS6 and MLA1/MLA6 receptors are functionally interchangeable when mediating cell death in *N. benthamiana*, although the corresponding MLA subfamilies 1 and 2 are mainly differentiated by this polymorphic N-terminal CC module. Recognition of AVR_{A1} and AVR_{A6} by SCS6-MLA hybrids required the presence of both NB and LRR domains from MLA1/MLA6 receptors. A hybrid receptor carrying MLA6 CC and NB domains and the SCS6 LRR stimulated cell death upon IWF_{ND90Pr} infiltration, although cell death activity was significantly weaker compared to WT SCS6 (M6LRRS6; Fig. 3b,c). However, when the LRR of MLA1 was exchanged with the SCS6 LRR (M1LRRS6), the resulting hybrid was nonresponsive to IWF_{ND90Pr} (Fig. S10), indicating that both SCS6 NB and LRR domains are involved in SCS6 activation by the Bs_{ND90Pr} nonribosomal peptide effector. All tested hybrid receptors accumulated to similar steady-state levels in *N. benthamiana* leaf tissue (Figs 3e, S10b). These findings suggest that a Bs_{ND90Pr}-released nonribosomal peptide effector specifically activates SCS6 via its LRR and NB domains.

Scs6 susceptibility to spot blotch is common in barley

In nature, direct activation of SCS6-mediated cell death might be a strategy for the spot blotch pathogen to sustain its necrotrophic growth phase on susceptible barley. Therefore, we investigated the prevalence of *Scs6*-mediated susceptibility in domesticated and wild barley (Fig. 4, Datasets S1, S2). We previously performed Bs_{ND90Pr} inoculation experiments with 1480 domesticated barley lines (Wang *et al.*, 2017). We extended this dataset by testing another 571 domesticated and 367 wild barley lines,

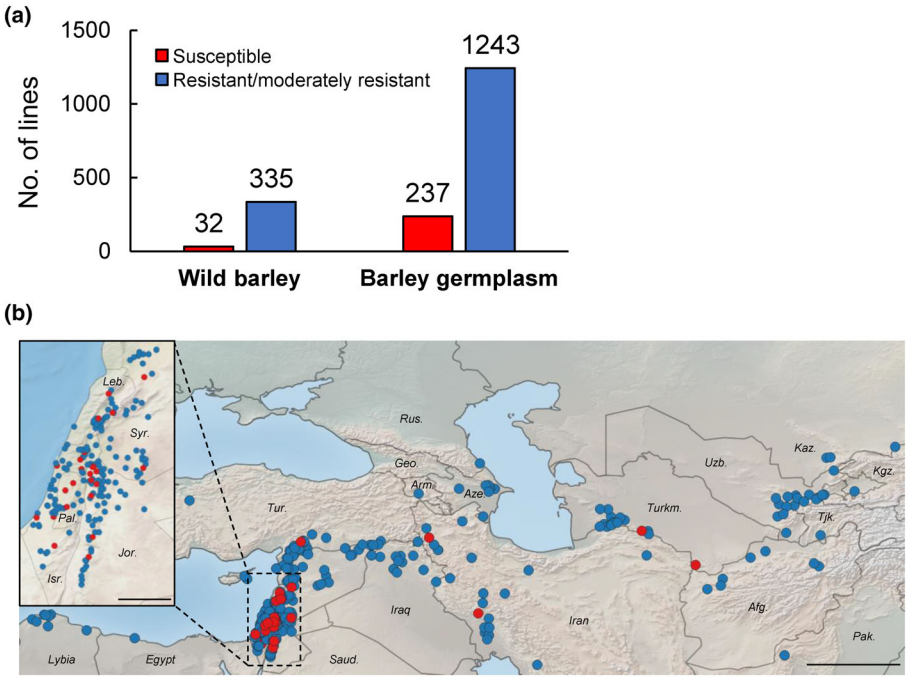

Fig. 4 *Scs6* susceptibility to spot blotch is common in wild and cultivated barley. (a) Summary of inoculation experiments of wild barley (*Hordeum spontaneum*) accessions, including accessions from the Wild Barley Diversity Collection (WBDC) (Roy *et al.*, 2010; Maekawa *et al.*, 2019), and a panel of *Hordeum vulgare* germplasm with *Bipolaris sorokiniana* isolate ND90Pr. (b) Geographic distribution of surveyed *Hordeum spontaneum* accessions. The 1–9 disease rating scale described by Fetch & Steffenson (1999) was used to rate the spot blotch disease after inoculation of ND90Pr. Plants with an average disease score of 1 to 3 were classified as resistant, 4 to 5 as moderately resistant, and 6 to 9 as susceptible. Susceptibility or resistance and moderate resistance to *B. sorokiniana* ND90Pr is indicated in red or blue, respectively. Scale: 500 km (large map) and 100 km (map section on the left). See also Supporting Information Datasets S1, S2.

the latter consisting of 318 accessions from the Wild Barley Diversity Collection (WBDC) and 49 additional *H. spontaneum* lines belonging to nine populations distributed throughout the Fertile Crescent (Roy *et al.*, 2010; Pankin *et al.*, 2018). Among the 346 susceptible domesticated barley accessions, we confirmed the presence of a *Scs6* allele by PCR in 234 lines tested. Of the latter, we additionally determined full-length *Scs6* sequences in 69 lines. In one of the resistant lines in which a *Scs6* allele was identified by PCR, DNA sequencing revealed that the gene encodes a truncated protein (Clho13653, 844 aa instead of 959 aa).

Thirty-two wild barley accessions were susceptible to Bs_{ND90Pr} (Fig. 4a). Based on targeted DNA sequencing of twenty accessions and seven previously sequenced wild barley accessions (Maekawa *et al.*, 2019), we verified that they encode closely related SCS6 haplotypes (> 97.90% protein sequence identity). FT170, for example, is highly susceptible and carries *FT170-1* as its sole subfamily 2 member, previously designated *Mla18-1* (Maekawa *et al.*, 2019). Barley line FT153 was clearly susceptible to Bs_{ND90Pr} although previously only one MLA subfamily 1 variant was annotated at its *Mla* locus (*FT153-1*) (Maekawa *et al.*, 2019), but the DNA sequencing of the corresponding genomic region detected a *Scs6* haplotype (*FT153-2*) that had escaped earlier analysis (Maekawa *et al.*, 2019). Thus, susceptibility of wild and domesticated barley to Bs_{ND90Pr} spot blotch is strictly linked to the presence of *Scs6*, identified here as a member of MLA subfamily 2.

The SCS6 receptor is likely a *Hordeum*-specific innovation

To investigate the evolutionary history of SCS6/MLA-mediated susceptibility to spot blotch, we curated a phylogenetic tree of all MLA variants found in wild and domesticated barley using neighbor-net analysis of full-length proteins. This revealed that SCS6 variants cluster within MLA subfamily 2 (Fig. 5a). In comparison to sequence divergence of individual MLA recognition specificities belonging to subfamily 1, sequence variation between SCS6 variants appear to be more limited although the corresponding accessions were sampled in distinct geographical regions and belong to different *H. vulgare subsp. spontaneum* populations (Fig. 5a). We examined an array of MLA subfamily 1 and subfamily 2 variants for sensitivity to IWF_{ND90Pr} in *N. benthamiana* and found that not only SCS6, but also subfamily 2 variants MLA16 and MLA18-1, can mediate effector-induced cell death and can therefore be considered SCS6 variants (Fig. 5b). However, sensitivity to the IWF was not shared among all MLA subfamily 2 members (e.g. MLA25, *Sc*Sr50; Fig. 5b–d). This shows that there is natural genetic variation among all available MLA subfamily 2 members that accounts for their differential sensitivity to the Bs_{ND90Pr} NPS1-derived effector as well as susceptibility to the pathogen.

We extended our aforementioned phylogenetic analysis, limited to *Hordeum* RGH1 variants, by including full-length proteins encoded by *Mla* orthologs or paralogs in other Triticeae species, including wheat (*Triticum*) and rye (*Secale*), and the wild grass *Dasypyrum villosum* (Fig. S14; Z. Huang *et al.*, 2022). MLA subfamilies 1 and 2 are mainly distinguished by their polymorphic CC domains (e.g. 65% identity and 81% similarity for MLA6 and SCS6 CC domains; Maekawa *et al.*, 2019). The CC domains of some MLA haplotypes present in *D. villosum* can be assigned to MLA subfamily 1, while others are assigned to MLA subfamily 2 (Fig. S15), indicating that the differentiation of the CC domain occurred before the speciation of barley and *Dasypyrum villosum*, that is *c.* 14.9 million years ago (Ma), which predates the divergence of wheat and barley 8 Ma (Zhang *et al.*, 2023). Notably, we did not identify SCS6 homologs in other grass species, suggesting that SCS6 is likely a *Hordeum*-specific innovation. We performed statistical analysis on the coding sequences of *Scs6* variants, MLA subfamily 2 members from barley, and other *Mla* subfamily 2 members in the Triticeae to identify sites under positive selection. Strong signatures of positive selection in the LRR domain of Triticeae *Mla* subfamily 2 members are consistent with the observation that some of them confer resistance to pathogens not only in barley but also in rye, such as *Sc*Sr50 (Fig. S16; Mago *et al.*, 2015; Maekawa *et al.*, 2019). SCS6 variants may be subject to purifying selection, which could explain their low-sequence diversity and weak signatures for diversifying selection.

Discussion

We have shown here that barley *Scs6* is necessary and sufficient to confer hyper-susceptibility to necrotrophic *B. sorokiniana* isolate Bs_{ND90Pr}. SCS6 is encoded at the complex *Mla* locus on chromosome 1H, which harbors three highly dissimilar but physically linked *NLR* families, *Rgh1*, *Rgh2* and *Rgh3* (Wei *et al.*, 1999, 2002). All characterized disease resistance specificities at this locus were exclusively assigned to the *Rgh1* family and a survey of wild barley revealed that *Rgh1* members are further sequence-diversified into two subfamilies, termed MLA subfamily 1 and subfamily 2 (Maekawa *et al.*, 2019). SCS6 shares 82% amino acid sequence identity with MLA6 and 28% and 24% sequence identity with RGH2 and RGH3, respectively, suggesting that a *bona fide* RGH1 member is needed for disease susceptibility of Bs_{ND90Pr}. Expression of barley *Scs6*, but not barley *Mla1* or *Mla6*, in evolutionarily distant *N. benthamiana* reconstitutes a cell death response, specifically triggered by IWF collected from barley plants infected by Bs_{ND90Pr} with an intact *VHv1* locus. Taken together with the capacity of autoactive $SCS6^{H502V}$ to mediate cell death in the absence of a pathogen effector and the fact that all resistant EMS mutants carry mutations in *Scs6*, this indicates that SCS6 acts as a singleton NLR activated by the NPS1-derived nonribosomal peptide effector. The deduced function of SCS6 as a virulence target of a necrotrophic pathogen contrasts with characterized immune receptors for biotrophic or hemibiotrophic pathogens encoded by *Rgh1*. In addition, only SCS6 is activated by a deduced small molecule, whereas all other RGH1 members are activated upon sensing proteinaceous pathogen effectors to confer immunity (Maekawa *et al.*, 2012; Lu *et al.*, 2016; Chen *et al.*, 2017; Saur *et al.*, 2019; Bauer *et al.*, 2021; Brabham *et al.*, 2023; Cao *et al.*, 2023).

Drastically reduced fungal biomass on barley *scs6* leaves compared to WT *Scs6* Bowman following inoculation with WT Bs_{ND90Pr} supports our conclusion that *Scs6* is a virulence target for the fungus. As the reduced fungal biomass is tightly linked to loss of infection-associated host cell death on *scs6* mutants, it raises the possibility that *Scs6*-triggered signaling and/or cell

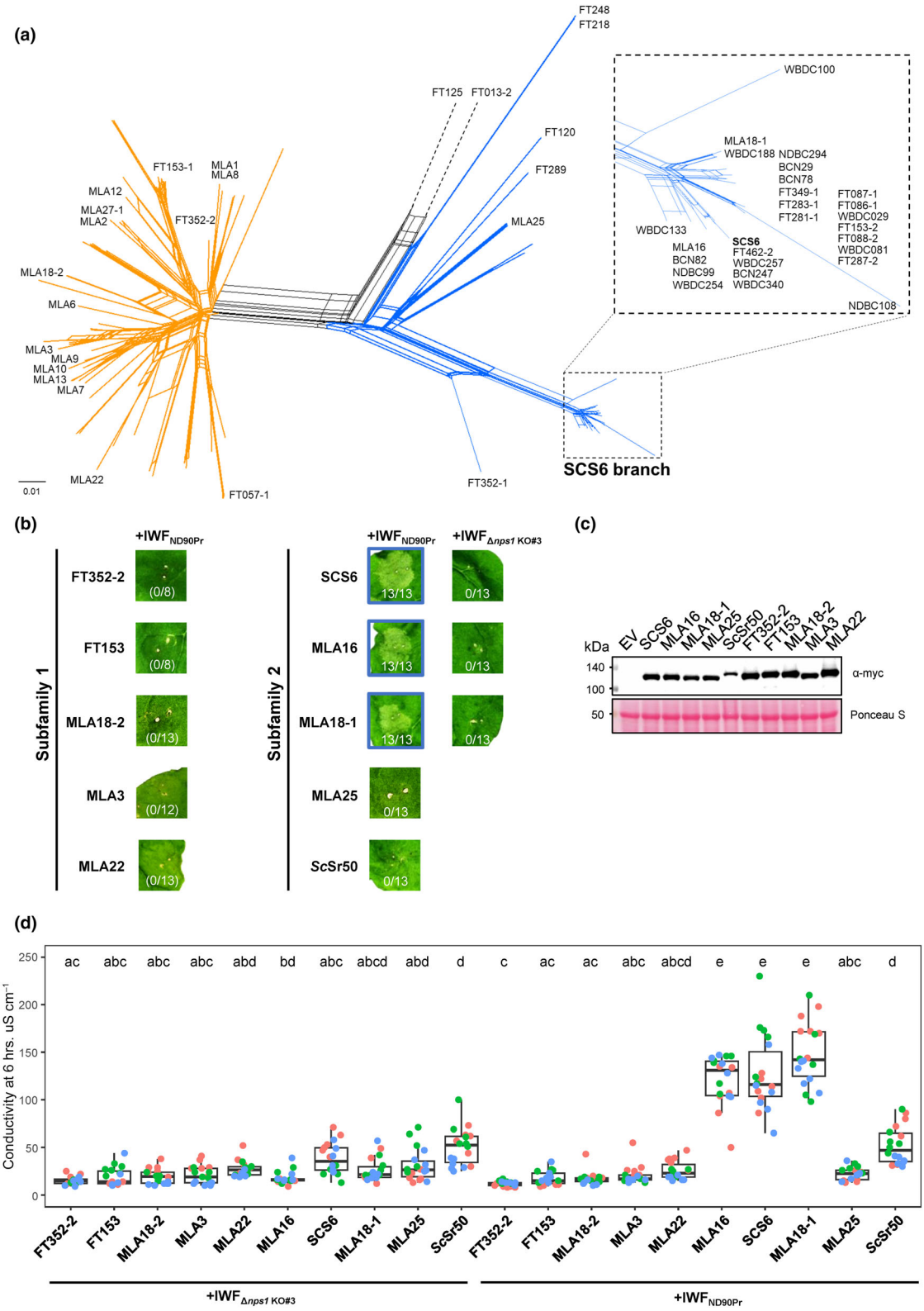

Fig. 5 Diversity at the barley MLA locus underlies differential sensitivity to the Bs_{ND90Pr} NPS1-derived effector as well as susceptibility to spot blotch. (a) Neighbor-Net analysis of 114 MLA protein sequences including 28 previously identified MLA proteins from barley (Seeholzer *et al*., 2010) 59 sequences from wild barley (Maekawa *et al*., 2019), as well as 27 sequences from wild or domesticated barley identified in this study. See also Supporting Information Datasets S3–S5. MLA subfamily 1 and MLA subfamily 2 members are represented using yellow or blue edges, respectively, based on (Maekawa *et al*., 2019) and Fig. S15. See also Fig. S17. Scale bar indicates size of network. No disease resistance activity has yet been identified for Subfamily 2 members *Mla16*, *Mla18* and *Mla25* (Maekawa *et al*., 2019). (b) *Nicotiana benthamiana* plants were transformed transiently, as indicated. Twenty-four hours after *Agrobacterium*-mediated gene delivery, IWF_{ND90Pr} or $IWF_{\Delta nps1\ KO\#3}$ was infiltrated, as indicated. Representative pictures of at least eight biological replicates (indicated in brackets) were taken 2 d after agroinfiltration and combinations that resulted in cell death are highlighted with a blue box. OD_{600} of *A. tumefaciens* was set to 0.5, except for *Sc*Sr50, for which the OD_{600} was reduced to 0.2 to attenuate auto-activity. (c) Protein accumulation levels of expressed receptor-4xmyc constructs were determined by α-myc Western blotting using total protein extracted from *N. benthamiana* leaves, 1 d after *Agrobacterium* infiltration. Western Blots are based on at least two replicates. (d) Ion leakage assays of cell death in *N. benthamiana* leaves after agroinfiltration of the indicated constructs and infiltration of IWF_{ND90Pr} or $IWF_{\Delta nps1\ KO\#3}$. Results were from three independent biological experiments (n = 18, 6 leaf disks for each experiment). Each individual dot represents one measurement and the colors indicate the corresponding biological replicate. In each box, the top, middle and lower horizontal lines correspond to the upper quartile, median and lower quartile values, respectively. Whiskers depict the maximum and minimum values, dots above or below the whiskers are being considered outliers. Letters indicate significant differences (analyzed by one-way ANOVA with Tukey's multiple comparisons test, adjusted P value < 0.05).

death promotes the necrotrophic lifestyle of the spot blotch pathogen. Two deduced NRPSs are encoded at the *VHv1* locus in the Bs_{ND90Pr} genome and are unique to pathotype 2 strains (Valjavec-Gratian & Steffenson, 1997b; Condon *et al*., 2013). Since deletion of one of the two *NRPS* genes at *VHv1* is sufficient to abolish high virulence of Bs_{ND90Pr} on cultivar Bowman (Condon *et al*., 2013), we propose that a nonribosomally encoded peptide effector produced by the fungus activates the SCS6 receptor. Our data obtained with transgenic barley show that *Scs6* is the only host factor needed to render resistant barley cultivars lacking this CNL hyper-susceptible to Bs_{ND90Pr}. This finding together with the observation that expression of barley *Scs6* is sufficient to reconstitute a cell death response in evolutionarily distant *N. benthamiana* in response to IWF_{ND90Pr} infiltration, suggest that SCS6 is likely the direct virulence target for the NRPS-derived effector. However, we cannot exclude the possibility that the NRPS-derived effector targets an unknown factor conserved in barley and *N. benthamiana*, which is needed for *Scs6* activation.

Besides direct binding of pathogen avirulence effectors to the LRR domain, plant NLR receptors can also indirectly sense effector-mediated modifications in host proteins that serve as virulence targets (Cesari, 2018; Burdett *et al*., 2019; Wang *et al*., 2019a,b). In such an indirect activation model for SCS6 one would expect the formation of a preactivation receptor complex through specific association with an unknown barley virulence target for the Bs_{ND90Pr}-derived effector. As *Scs6* is shown here to be a lineage-specific innovation in barley (*Hordeum*), it seems unlikely that a preactivation SCS6 complex can assemble in heterologous *N. benthamiana*, as this would imply an exceptional degree of evolutionary conservation of a hypothetical virulence target between dicotyledonous and monocotyledonous plants – species that diverged from each other *c*. 140 Ma (Chaw *et al*., 2004). Thus, our results contrast with the indirect recognition of the victorin toxin by the LOV1 CNL of *A. thaliana* through victorin-mediated disruption of *At*TRXh5 activity (Lorang *et al*., 2012). In agreement with our conclusion, neither the expression of *LOV1* nor *AtTRXh5* alone in *N. benthamiana* leaves is sufficient to induce cell death after victorin infiltration (Lorang *et al*., 2012). If *Vb*/*Pc-2* in oat is the same gene and encodes an NLR (Mayama *et al*., 1995; Wolpert & Lorang, 2016), it will be interesting to test whether this receptor from the natural host of *C. victoriae* is directly or indirectly activated by victorin. Finally, the reconstitution of IWF-triggered and barley SCS6-dependent cell death in heterologous *N. benthamiana* suggests that the Bs_{ND90Pr} NPS1-derived effector can enter plant cells in the absence of pathogen infection structures and in the absence of a potential host species-specific surface receptor or transporter.

Similar to the proposed function of SCS6 as a direct virulence target for the Bs_{ND90Pr} NPS1-derived effector, experimental evidence, including structural data from an MLA-effector complex (Lawson *et al*., 2024), suggests that several other characterized barley RGH1 CNLs directly bind to proteinaceous avirulence effectors delivered by biotrophic *B. graminis* f sp *hordei* via the polymorphic LRR for receptor activation. These include MLA7, MLA10, MLA13, and MLA22, which respectively bind to sequence-diversified avirulence effectors AVR_{A7}, AVR_{A10}, AVR_{A13} and AVR_{A22} that share a common structural scaffold (Saur *et al*., 2019; Bauer *et al*., 2021; Cao *et al*., 2023). Similar to SCS6, matching pairs of these MLA receptors and AVR_A effectors are necessary and sufficient to induce a cell death response in heterologous *N. benthamiana*. Additionally, the CNL receptor encoded by the stem rust resistance gene *ScSr50* in wheat, an orthologue of barley *Rgh1* derived from rye chromosome 1R, assigned here to MLA subfamily 2, appears to bind directly to the stem rust effector AvrSr50 (Chen *et al*., 2017; Ortiz *et al*., 2022). Collectively, this indicates that RGH1 CNLs may have a propensity to interact directly with structurally distinct proteinaceous and even specialized nonribosomal peptide effectors.

One of the EMS-induced mutants encodes a receptor variant with a single amino acid substitution in the LRR domain, $SCS6^{S793F}$, which results in both loss of susceptibility to Bs_{ND90Pr} in barley and loss of cell death activity in response to IWF_{ND90Pr} infiltration in *N. benthamiana* (Figs 1, 3). Based on an AlphaFold2-generated SCS6 model, the residue S793 has an outward-facing side chain and is located on the concave side of the LRR. This, together with our observation that the SCS6 LRR

domain is sufficient to confer IWF responsiveness to the corresponding MLA6 hybrid receptor M6LRRS6, corroborates an essential role of the SCS6 LRR as direct virulence target for the fungal-derived NRPS effector. By contrast, in the Bs_{ND90Pr}-resistant barley mutants EMS14 and EMS1473, the deduced inward-facing receptor residues S73 and L183 are substituted by bulky phenylalanine, which is expected to destabilize the conformation of the CC and NB-ARC domains, respectively. If SCS6 functions similarly to sensor CNLs Sr35 and ZAR1 in wheat and Arabidopsis, then the latter two single amino acid substitutions in the SCS6 receptor might abolish the virulence activity of SCS6 by interfering with receptor oligomerization or Ca^{2+} pore formation after binding of the effector to the SCS6 LRR domain (Bi *et al.*, 2021; Förderer *et al.*, 2022a). In addition to the LRR, the NB domain was found to contribute to the specific targeting of SCS6 by the peptide effector (Fig. S10), suggesting that the effector might interfere with NB and LRR interdomain interactions for receptor activation.

Although *B. sorokiniana* isolates are typically generalists that can infect a wide range of Triticeae species, including wheat, the isolate Bs_{ND90Pr} is specialized to barley hosts. This is consistent with our finding that *Scs6* alleles were not detected in wheat or wheat progenitors, suggesting that *Scs6* might be a *Hordeum*-specific innovation that evolved after the divergence of the genera *Triticum* and *Hordeum* < 8 Ma (Middleton *et al.*, 2014). This could explain why Bs_{ND90Pr} confers hyper-susceptibility only on *Scs6* barley genotypes, raising the possibility that *Bs* pathotype 2 acquired its unique *VHv1* virulence gene cluster during interactions with *Hordeum* hosts. However, whether *VHv1* of Bs_{ND90Pr} evolved as a postdomestication event in agricultural environments or in wild barley pathogen populations and subsequently spread to North America remains to be clarified.

All characterized *Mla* powdery mildew disease resistance specificities in barley belong to *Mla* subfamily 1, whereas no disease resistance function has yet been assigned to barley *Mla* subfamily 2, which includes *Scs6*. Extensive data support the notion that functional diversification of MLA subfamily 1 members is driven by a coevolutionary arms race with the genetically highly diverse biotrophic *Bgh* pathogen (Spanu *et al.*, 2010; Pedersen *et al.*, 2012; Frantzeskakis *et al.*, 2018; Maekawa *et al.*, 2019). Compared to MLA subfamily 1 members, our sequence analysis of subfamily 2 members shows low-sequence diversity and weak evidence for diversifying selection, if any. SCS6 is maintained in several wild barley populations with an incidence of *c.* 8%, suggesting a beneficial function for the host. For comparison, the incidence of MLA subfamily 1 members conferring known powdery mildew resistance in wild barley may be well below 8%, as none of the 13 molecularly characterized MLA resistance specificities were found in 50 wild barley accessions representing nine populations (Seeholzer *et al.*, 2010; Maekawa *et al.*, 2019). Thus, the widespread occurrence of *Scs6* and the ability of autoactive $SCS6^{H502V}$ to trigger cell death in the absence of a pathogen effector, makes it likely that the SCS6 CNL confers a fitness benefit against unknown biotrophic or hemibiotrophic pathogens endemic to barley populations in the Fertile Crescent, by acting as sensor NLR. In contrast to the powdery mildew fungus, the postulated pathogen does not currently appear to be engaged in a rapid coevolutionary arms race with extant *Hordeum spontaneum* germplasm.

The hyper-virulent Bs_{ND90Pr} isolate emerged 6 yr after barley cultivar Bowman was introduced in North Dakota in 1985. Unexpectedly, our pathotyping survey shows that *Scs6*-dependent susceptibility to Bs_{ND90Pr} is twice as high in domesticated barley as in wild barley populations (16% and 8%, respectively). Domestication and breeding for disease resistance in barley may have inadvertently resulted in the co-enrichment of *Scs6*-dependent disease susceptibility to Bs_{ND90Pr}, probably due to linkage drag from another disease resistance gene on barley chromosome 1H. Recently, the *Pyrenophora teres* f. *maculata* susceptibility factor *Spm1* was mapped to the *Mla* locus in the cultivar Baudin (Muria-Gonzalez *et al.*, 2023). Although it remains to be tested whether *Spm1* is also a member of the *Rgh1* family, our results demonstrate that the evolution of allelic variants of a single *R* gene is shaped by contrasting selective pressures exerted by multiple pathogens with different lifestyles. Elucidating the molecular principles underlying SCS6 activation by the NPS1-derived effector is likely to be of broader importance, as this could aid future development and deployment of synthetic NLR receptors in crops that are less vulnerable to manipulation by economically important necrotrophic pathogens.

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Competing interests

None declared.

Author contributions

YL, FK, PS-L and SZ designed research; YL, FK, MZ, IM, EL, PK, PX, SY, MJM and SM performed research; JD, JDF, YD, BS, SM and BJS contributed new reagents/resources/analytic tools; YL, FK, SY, PS-L and SZ analyzed data; and FK, YL, PS-L and SZ wrote the paper. All reviewed the manuscript. YL and FK contributed equally to this work.

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Data availability

The data that supports the findings of this study are available in the Supporting Information of this article (Datasets S1–S5, Figs S1–S17 and Tables S1–S4).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Cultivated barley (*Hordeum vulgare* subsp. *vulgare*) lines used in this study and their reaction to the isolate ND90Pr of *Bipolaris sorokiniana*.

Dataset S2 Wild barley (*Hordeum vulgare* subsp. *spontaneum*) lines used in this study and their reaction to the isolate ND90Pr of *Bipolaris sorokiniana*.

Dataset S3 Accession identifiers for proteins used in the phylogenetic analysis.

Dataset S4 Fasta-sequences of proteins used in phylogenetic analysis (excluding outliers).

Dataset S5 Codon-aware multiple-sequence alignment of Subfamily 2 members in the Triticeae.

Fig. S1 Infection responses of wild-type barley cv. Bowman, Golden Promise (GP), DH47, and representative Scs6 transgenic barley lines ($GP_{T1\text{-}3}$ and $DH47_{04659\text{-}8\text{-}4}$) from GP and DH47 to isolate ND90Pr and two independent NPS1 mutants (Δnps1 KO#3 and KO#9) of *Bipolaris sorokiniana*.

Fig. S2 Gene constructs used for transformation of barley cv. Golden Promise and barley line SxGP DH-47.

Fig. S3 Chromosome flow sorting of 1H.

Fig. S4 PCR analysis of *Scs6* transgenic barley plants.

Fig. S5 Relative expression of *Scs6* in different barley genotypes at different time points after inoculation with the wild-type isolate ND90Pr or the NPS1 knockout mutant (Δ*nps1* KO#3).

Fig. S6 Partial characterization of the *Bipolaris sorokiniana* isolate ND90Pr NPS1-derived effector.

Fig. S7 Whole leaf pictures of *Mla6*/*Scs6* chimeric receptors co-expressed with AVR_{A6}.

Fig. S8 Whole leaf pictures of *Mla6*/*Scs6* chimeric receptors expression and subsequent IWF_{ND90Pr} infiltration.

Fig. S9 Whole leaf pictures of *Mla6*/*Scs6* chimeric receptors co-expressed with an empty vector control.

Fig. S10 A *Bipolaris sorokiniana* ND90Pr effector activates SCS6 to cause cell death in *N. benthamiana* depending on its nucleotide-binding domain and leucine-rich repeat region domain.

Fig. S11 Whole leaf pictures of *Mla1*/*Scs6* chimeric receptors co-expressed with AVR_{A1}.

Fig. S12 Whole leaf pictures of *Mla1*/*Scs6* chimeric receptors expression and subsequent IWF_{ND90Pr} infiltration.

Fig. S13 Whole leaf pictures of *Mla1*/*Scs6* chimeric receptors co-expressed with an empty vector control.

Fig. S14 Phylogenetic tree including RGH1 sequences identified in members of the Triticeae.

Fig. S15 Phylogenetic tree of coiled-coil domains of RGH1 members identified in members of the Triticeae.

Fig. S16 Identification of sites under positive selection in SCS6 and all MLA subfamily 2 members from the Triticeae.

Fig. S17 Maximum-Likelihood phylogenetic tree of RGH1 protein sequences shown in Fig. 5(a).

Table S1 Primers used in this study.

Table S2 Summary of chromosome 1H sorting and sequencing of wild-type and EMS mutants.

Table S3 Mutation overlap in contigs from flow-sorted 1H chromosome of barley.

Table S4 Infection responses and PCR amplification of the transgenic barley individuals derived from barley line SxGP DH47 to *Bipolaris sorokiniana* isolate ND90Pr.

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