Physiological and Molecular Plant Pathology 85 (2014) 53–64

Contents lists available at ScienceDirect

Physiological and Molecular Plant Pathology

journal homepage: www.elsevier.com/locate/pmpp

Differential responses of *Brachypodium distachyon* genotypes to insect and fungal pathogens

Germán V. Sandoya [a,b], Marcia Maria de Oliveira Buanafina [a,*]

[a] Department of Biology, 208 Mueller Laboratory, The Pennsylvania State University, University Park, PA 16802, USA
[b] University of California, Davis, USDA-ARS, The Genome Center, Department of Plant Sciences, 1636 E Alisal St., Salinas, CA 93905, USA

ARTICLE INFO

Article history:
Accepted 22 January 2014

Keywords:
Brachypodium distachyon
Magnaporthe grisea
Sclerotinia homoeocarpa
Rhizsoctonia solani

ABSTRACT

To increase understanding of the interactions between *Brachypodium distachyon* (purple false brome) and its pathogens, six diploid and two hexaploid Plant Introductions (PI) lines were assessed for their resistance/susceptibility to nine economically important fungal pathogens and two species of insect pests affecting closely related grass species. Naturally occurring variation in resistance was found, with two lines being the most resistant and one line being the most susceptible to most of the insects and pathogens tested. Evidence was found for differential activation of key genes in pathogen defense response pathways between susceptible and resistant lines.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Studies of the molecular biology and genetics of plant resistance have provided much information on how plants recognize and fight invading organisms. The use of *Arabidopsis thaliana* as a model plant in such studies has made a major contribution to our understanding of plant–biotic stress interactions [1]. However, *A. thaliana* is not an ideal model organism for testing responses of grasses to pests and pathogens. For one thing, its cell wall architecture is completely different from that found in grasses. Since the cell wall is the plant's first barrier to invading insects or pathogens, this is an important consideration.

Brachypodium distachyon (L) Beauv., purple false brome, is a winter annual grass (Poaceae) being widely used as a model plant for temperate grasses because of its small genome, short life cycle, and small size [2,3]. It has been proposed for use as a model in studies of specific plant–pathogen or plant–pest interactions involving rice [4,5], wheat [5–8], and barley [8]. However, there are many cultivated grasses important in the turfgrass, animal, and biofuel industries, among others, for which plant–pathogen/pest interaction models still need to be established. If *Brachypodium* is to be used as a model, much more information about the pathogen and pest responses of different genotypes of *Brachypodium* is needed.

The Poaceae family—which includes crucial cultivated crops such as wheat, maize, rice, and barley—can be attacked by several serious insects and pathogens. Major insect pests include the fall armyworm (FAW, *Spodoptera frugiperda* Smith), a "tissue-feeding" insect that attacks a wide variety of grasses [9], and "phloem-feeding" insects such as the Russian wheat aphid (RWA, *Diuraphis noxia* Kurdjumov) [10], which has dramatically reduced the yield of wheat and barley in the United States since its invasion in 1986.

Pathogens on grasses cause a wide range of serious problems including blast disease [11,12], foliar disease [13,14], seed rot [15], damping off [15,16], "dollar spot" [17], and root necrosis [18–20]. In addition to damaging or killing crops, fungal pathogens may also produce metabolites such as mycotoxins that are harmful to humans and livestock that consume affected plant tissue.

Resistance mechanisms in grasses are complex and may involve constitutively expressed primary and secondary metabolites and induced metabolites such as phytoalexins, ethylene, and H_2O_2 accumulation [21–23]. The metabolites jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) are produced in response to attack by insects or pathogens [24,25]. These metabolites aid plant defense in a variety of ways. For example, ethylene (ET) elicits H_2O_2 production, and along with peroxidase (POX2) leads to increased ferulate dimerization and strengthening of the cell wall, enhancing the wall's function as a barrier to potential invaders.

Plants also produce reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to the production of pathogenesis-related (PR) proteins [26]. Among PR proteins, peroxidases have

Abbreviations: FAW, fall armyworm; RWA, Russian wheat aphid; SA, salicylic acid; JA, jasmonic acid; ET, ethylene; ACC, 1-aminocyclopropane-1-carboxylic acid; 4CL, 4-coumarate:CoA ligase; COI1, coronatine-insensitive protein 1; LOX3, lipoxygenase 3; NPR1, non-expressor of pathogenesis-related genes 1; POX2, peroxidase; SAM-DC, S-adenosyl methionine.

* Corresponding author. Tel.: +1 814 8670223.
E-mail addresses: gsandoya@ucdavis.edu (G.V. Sandoya), mmb26@psu.edu (M.M.deO. Buanafina).

0885-5765/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.pmpp.2014.01.001

been shown to be effective against pathogens [27] and insect attack [28–31]. Class III plant peroxidases are known to participate in a broad range of physiological processes, such as lignin and suberin formation and cross-linking of cell wall components [26].

In this study, we compare the resistance/susceptibility of eight *Brachypodium* lines to nine economically important fungal pathogens (*Magnaporthe grisea*, *Colletotrichum cereale*, *Sclerotinia homoeocarpa*, *Pythium aphanidermatum*, *Rhizoctonia solani*, *Magnaporthe poae*, *Ophiosphaerella agrostis*, *Ophiosphaerella korrae*, and *Gaeumannomyces graminis*) and two insect pests (fall armyworm, *S. frugiperda*, and Russian wheat aphid, *D. noxia*) affecting closely related grass species. We found natural variation in resistance among the *Brachypodium* genotypes. We further provide evidence for differential activation between susceptible and resistant accessions of key genes of response pathways that are often activated during pathogen attack.

2. Material and methods

2.1. Plant material

Eight *B. distachyon* accessions of different origin and ploidy levels, as listed in Table 1, were included in this study. Two of these were the sequenced PI Bd21 and the inbred line derived from this PI, Bd21-3 [2]. Seeds were supplied by Vicki L. Bradley, USDA, ARS, WRPIS, Washington State University.

2.2. Pathogen screening of B. distachyon genotypes

2.2.1. Plant growth conditions

Seeds of the eight *B. distachyon* accessions were germinated on humidified paper towels in 24-well cell culture plates from CellStar®. Germinated seeds were transferred to 3.5″ or 4.5″ diameter pots (depending on the experiment) containing 5:1 mixture of Miracle-Gro Potting Mix and vermiculite. These seeds were grown in a controlled greenhouse at 24/18 °C (day/night) temperature and a 16-h photoperiod, for six to eight weeks prior to FAW screening, except for genotype CWC, which was grown for four weeks. Plants were fertilized at two-week intervals with a solution containing 1.1 g l^{-1} N, 0.28 g l^{-1} P and 1.0 g l^{-1} K and watered regularly.

2.3. Insect performance on Brachypodium leaves

2.3.1. FAW—source of insects

Eggs of the maize host strain of the fall armyworm (FAW, *S. frugiperda*) were supplied by the USDA-Corn Insect Research Facility at Mississippi State University, through Dr. Dawn Luthe (PSU). Newly hatched caterpillars were reared on a wheat germ- and casein-based diet [32]; ingredients were purchased from BIOSERV (Frenchtown, NJ, USA). Caterpillars were kept individually in diet cups in an incubator at 27 °C and 16 h of light [33] for approximately four days, until they reached their second instar, before being used for bioassays. Bioassays were conducted with larvae of approximately the same stage and weight.

Table 1
Brachypodium distachyon genotypes used in this research, nomenclature, plant introduction (PI) accession number, geographic origin and ploidy level.[a]

Nomenclature used in this research	PI accession	Geographic origin	Ploidy
CWA	Bd[b] 21-3	Iraq	Diploid
CWB	Bd 21	Iraq	Diploid
CWC	227011	Iran	Hexaploid
CWD	372787	Uruguay	Hexaploid
CWE	185133	Iraq	Diploid
CWF	245730	Turkey	Diploid
CWG	208216	South Africa	Diploid
CWJ	Bd 3-1	Iraq	Diploid

[a] Vogel et al. [3].
[b] Bd indicates an inbred.

2.3.2. FAW—larval growth performance

Second-instar larvae were placed on one of seven particular diet treatments, held in individual diet cups containing 1% agarose (to avoid drying of the leaf material). Diet treatments consisted of fully developed leaves of the different *B. distachyon* genotypes, assayed in one, two, or three trials, depending on the genotype. For each treatment, 15 larvae were used. Larvae were allowed to feed on fresh leaf tissue, added every other day according to the caterpillars' needs, for ten days. Diet cups were cleaned daily to avoid fungal growth. Cups containing larvae and food were held under laboratory conditions, at 21 °C and a 10:14 day/night photoperiod. Larvae were weighed at the start of the experiment and every other day afterwards to follow their development on the different *Brachypodium* genotypes and on control (maize and fescue) leaves. The data presented here were obtained ten days after starting the bioassay, when FAW caterpillars reached their highest larval weight, and just before pupation on the control host plants (susceptible maize inbred B73) under the experimental conditions.

2.3.3. Control plant growth conditions

Maize (*Zea mays*) inbred lines Mp708, Tx601 and B73—which have been reported previously as resistant, medium susceptible, and highly susceptible, respectively, to the FAW under Pennsylvania conditions [34]—were used as controls, along with *Festuca arundinacea* genotype (20BN3) from the cultivar S170. Maize plants were grown in a Hagerstown loam in a greenhouse with 16 h of light at a temperature of 28/22 °C (day/night) until reaching V7 to V8 stage (four to five weeks after planting); the mid-whorl part of the plant was used for the bioassays. Tall fescue tillers were grown in a controlled environmental chamber at 22/16 °C (day/night) temperatures and a 16-h photoperiod.

2.3.4. Aphids—source of insects

The two biotypes of Russian Wheat Aphid (RWA, *D. noxia*) RWA1 and RWA2 were supplied by the USDA-ARS, Stillwater, OK. These biotypes, previously described as the least (RWA1) and most (RWA2) virulent of this species [10], were maintained individually on the susceptible wheat variety "Yuma" for colony multiplication and insect development. The experiment was set up in cages specifically built for this purpose, using the insect screen LS Econet, from L. Svensson, Inc.®, with light transmission of 84% and 600 × 600 mm hole size. Cages measured 41 cm (L × W × H).

2.3.5. RWA—growth performance

Two replicate (4.5″) pots were used to grow each *Brachypodium* genotype (CWA-CWJ) and wheat control (Yuma) line, and plants were randomly arranged in cages (76 × 50 × 46 cm, $L \times W \times H$) that were placed inside a growth chamber for the duration of the experiment. Plants were fertilized and watered regularly, as previously described. Infestation was made with ~10 aphids, a mix of nymphs and adults, per plant. The experiment was evaluated when wheat plants reached level 8–9 of chlorosis (plants completely attacked), which in this experiment occurred after two weeks. The evaluation was made by visual rating using a scale from 1 to 9, where a rating of 1 was given to a healthy plant and a rating of 9 to a completely dead or leaf-rolling plant [35,36]. However, leaf rolling was rarely observed in these experiments.

2.4. Fungal pathogen performance on Brachypodium leaves

2.4.1. Sources of pathogens

The selected classes of fungi for these experiments—such as *M. grisea*, *C. cereale*, *R. solani*, *S. homoeocarpa*, *P. aphanidermatum*, *O. agrostis*, *O. korrae*, *G. graminis* var. *avenae*, and *M. poae*—were based on their pathogenic relevance to cultivated grasses as well as their relevance to the turf-grass industry. Plants were grown in 4″ diameter pots in a controlled greenhouse environment, as described above, for four to five weeks prior to screening with the different pathogens. All screening experiments were carried out in a second greenhouse under a 16-h photoperiod. High-pressure sodium lamps were used to simulate summer conditions.

2.4.2. Fungal screening

2.4.2.1. M. grisea (rice blast disease). Five isolates—named 4091-5-8, supplied by Dr. Seogchan Kang; Guy 11; IE1K; 18-1; and IA45, supplied by Dr. Yinong Yang (see Table 2)—were grown in oatmeal agar plates for approximately ten days prior to inoculation. Oatmeal agar plates were prepared using fifty grams of rolled oats heated in 500 ml of water for 1 h. Material was filtered through cheesecloth and the volume adjusted to 1000 ml with water. Glucose (1%) and agar (1.5%) were added and the suspension autoclaved at 121 °C for 35 min. Conidia were collected with a solution of Tween 0.01% in distilled, autoclaved water and the concentration adjusted to 200,000 spores/ml^{-1}. Approximately 1 ml of inoculum was used to spray individual plants using a 3345 VWR adjustable spray PK3 bottle (Control Company). Following inoculation, each plant was covered with a transparent plastic bag for 48 h to create high humidity and temperature for the normal development of the fungus. After a 48-h period, the plastic bags were removed and plants were kept under greenhouse conditions (as described above) for no longer than a week. Disease incidence was recorded daily from 72 h after infestation, for a period of one week, using a rating scale from 0 to 5, where 0 indicated no visible evidence of infection and 5 corresponded to large eyespot lesions, following Valent et al. [37] and adapted to *B. distachyon*.

2.4.2.2. C. cereale (Anthracnose disease, formerly C. graminicola). Isolate 246 CgMa-1, supplied by Dr. John Kaminski (see Table 2), was grown in Potato Dextrose Agar (PDA) media for approximately two weeks prior to inoculation. PDA was prepared with 300 g of fine slices of unpeeled potatoes in 1000 ml of water and heated for 40 min. The suspension was then filtered using cheesecloth and adjusted to 1000 ml with water. 2% dextrose and 1% agar were added and autoclaved for 35 min at 121 °C. Prior to inoculation, the conidia concentration was adjusted to 400,000 spores/ml^{-1} and plants infected as for *M. grisea*. Disease symptoms appeared after 96 h of infection as small patches on the leaf, and evaluations were made from that point onwards for a period of one week. Disease development was recorded using a rating scale from 0 to 5, where 0 represented no infected leaves, 1 indicated the presence of chlorotic flecks, and 5 represented a plant showing necrotic lesions (adapted from Ref. [38]).

Table 2
Insect species and fungal isolates used in this research.

Organism		Biotypes/isolates	Source of isolates
Insects	Fall Armyworm (*Spodoptera frugiperda*)	Corn biotype	Dr. Dawn Luthe (PSU)
	Russian Wheat Aphid (*Diuraphis noxia*)	RWA1, RWA2	Dr. Gary Puterka (USDA-ARS, Stillwater, OK)
Fungi	*Magnaporthe grisea*	40901-5-8 Guy 11, IE1K, IA45, 18-1	Dr. Seogchan Kan (PSU) Dr Yinong Yang (PSU)
	Colletotrichum cereale	246 CgMA-1	Dr. John Kaminski (PSU)
	Rhizoctonia solani	RhBC 2-1-3	Dr. Wakar Uddin (PSU)
	Pythium aphanidermatum	P3	Dr. Waker Uddin (PSU)
	Ophiosphaerella agrostis	81OpMO4, 548 OKCT-3	Dr. John Kaminski (PSU)
	Ophiosphaerella korrae	548 OKCT-3	Dr. John Kaminski (PSU)
	Gaeumannomyces graminis var. *avenae*	389 GGa CT-T	Dr. John Kaminski (PSU)
	Magnaporthe poae	626 MpNY-2	Dr. John Kaminski (PSU)
	Sclerotinia homoeocarpa	S-00-5044B	Dr. Waker Uddin (PSU)
	Sclerotinia homoeocarpa	241 ShMp-2	Dr. John Kaminski (PSU)

2.4.2.3. R. solani (Sheath blight). Isolate RhBC 2-1-3 (see Table 2), supplied by Dr. Wakar Uddin, was grown on PDA media for 10 days at 22–24 °C under continuous light. Small agar plugs were then transferred to autoclaved PDB (Potato Dextrose Broth) medium under aseptic conditions and cultured in the dark for 10 days with continuous shaking (260–280 rpm) at 28 °C. Fungal mycelia were harvested and cut into small (~5 mm) ball-shaped segments and attached at the lower sections of the stems, around the sheath of the *Brachypodium* plants. Inoculums were covered with aluminium foil for about 48 h, as this was the time period after which signs of the disease appeared in rice. Evaluations were made one and two weeks after inoculation using a scoring scale from 0 to 5, where 0 indicated no lesions and 5 indicated necrosis across the entire leaf section, resulting in leaf death [39].

2.4.2.4. S. homoeocarpa (Dollar spot). Isolate S-00-5044B, supplied by Dr. Wakar Uddin, and isolate 241 ShMp-2, supplied by Dr. John Kaminski (see Table 2), were directly cultivated on PDA for a week. An agar plug (~5 mm diameter) was directly inoculated around the stems of different tillers of *Brachypodium* plants and covered with aluminium foil to avoid drying and to create a suitable environment for fungal development. The aluminium foil was removed after 48 h, when the first signs of the disease were observed, and evaluations started 72 h after inoculation and continued for a period of one week. An arbitrary rating scale from 0 to 5 was used, where 0 indicated no disease and 5 a completely wilted brown plant.

2.4.2.5. P. aphanidermatum (Pythium blight). Isolate P3, supplied by Dr. Wakar Uddin (see Table 2), was grown on PDA media for approximately one week before inoculation. Inoculations were carried out by placing an agar plug with growing mycelia directly on the plant's stem, close enough to the soil to induce the disease in both foliar and root tissues. The agar plugs were covered with a piece of aluminium foil to avoid drying. The progression of the disease was scored a week after inoculation on a scale from 0 to 5, where 0 indicated no sign of disease and 5 a completely wilted plant.

O. agrostis (isolate 81 OpMO4), *O. korrae* (isolate 548 OKCT-3), *G. graminis* var. *avenae* (isolate 389 GGa CT-T), and *M. poae* (isolate 626 MpNY-2) were supplied by Dr. John Kaminski (see Table 2) and were grown in PDA plates for approximately one to two weeks, depending on the pathogen. Agar plugs were transferred to oat seeds (which had been first washed with tap water until completely clean, soaked in water overnight, and autoclaved for 1 h at 20 °C, on two consecutive days), and cultures were grown for a further two to three weeks under laboratory conditions (at ~21 °C) and under continuous white light supplied by a 15 V fluorescent lamp. Inoculations were made using 0.3 g of infected seeds (approximately 10 seeds) by placing the seeds directly into the soil, in contact with *Brachypodium* roots. Disease symptoms were observed two to three weeks after inoculation and evaluations

made using a rating scale from 0 to 10, with 0 being no sign of disease and 10 being plants that were completely dead [20].

2.5. Statistical analyses

Individual experiments for FAW and RWA were analysed as a Randomized Complete Design (RCD). Performance of *Brachypodium* genotypes against the different pathogens was tested individually for each pathogen and isolate – as a Randomized Complete Block Design (RCBD), with two or three replicates depending on the experiment. The *Brachypodium* genotypes were considered as treatments, using ANOVA with a PROC GLM of SAS Institute V.S. 9.2 (SAS Institute, 2010) [40]. In each type of experiment, means were separated using the protected LSD (Least Significant Difference) of Fisher ($p \geq 0.05$), and as such the test was applied only when the *F*-value was significant in the ANOVA [41].

2.6. Determination of resistance factors

2.6.1. Peroxidase activity

Peroxidase activity (POX) was determined in 50 mg of leaf tissue, collected at different time points following inoculation with different pathogens (see Table 2). Harvested samples were ground to a fine powder in liquid nitrogen, with a 2000 Geno/Grinder machine (SPEX SamplePrep®), and total protein extracted with 0.1 M potassium phosphate buffer, pH 7 and PVPP (5%). Following protein extraction, samples were placed on ice for 5 min, centrifuged at 4 °C at 11,000 g for 10 min, and the supernatant used to test for POX activity by following the change of guaiacol polymerization [42]. Twenty five μl of sample, 926 μl of 3 mM guaiacol, and 49 μl of 1 mM hydrogen peroxide were used and the change in absorbance at 450 nm determined over a period of 5 min in a Thermo/Spectronic 21D Milton Roy® spectrophotometer. Protein concentrations were determined according to Bradford [43], with bovine serum albumin as the standard. POX activity was expressed as nmoles mg protein^{-1} min^{-1}

2.6.2. Gene expression

Based on their well-known role in response to pathogen attacks in wheat, barley, and rice, key genes involved in the JA, ET, phenylpropanoid (PP), and SA pathways and peroxidase (Table 3) were chosen for gene-expression studies. Respective gene sequences were selected from the National Centre for Biotechnology Information (NCBI) website database and blast searched against the *Brachypodium* database (www.brachypodium.org). The most significant cDNA sequences were used to design primers for quantitative real-time (qRT) PCR. In addition, SamDC (*S*-adenosyl methionine decarboxylase) was selected as a housekeeping gene [44] to normalize the Cycle Threshold (Ct) values of the genes of interests (GOIs). Primers were designed with Primer Express Software® from Applied Biosystems, using default settings, and checked for single-peak amplification by RT-PCR prior to their use in experiments. Following identification of genotypes for either resistance or susceptibility to the different pathogens, three experiments were set up in order to study the transcript levels of the genes listed in Table 3, following infection with three different pathogens. In the first experiment, genotypes CWB and CWF were inoculated with *S. homoeocarpa* isolate S-00-554B. In the second experiment, genotypes CWC and CWG were analysed following inoculation with *O. agrostis* isolate 81 OpMO4. In the third experiment, CWC and CWF genotypes were infected with *M. grisea* isolate 4091-5-8. In each experiment, 100 mg of leaf tissue was collected before (control) and then 24 and 48 h after inoculation with *S. homoeocarpa*; 48 and 96 h following inoculation with *O. agrostis*; and after 24 and 48 h with *M. grisea*. Samples were ground in liquid nitrogen using a GenoGrinder machine (OPS Diagnostics LLC, Lebanon, NJ), and RNA was isolated from plant tissue using TRIzol® (Invitrogen, Carlsbad, CA), following the published online protocol (www.invitrogen.com). Samples were subsequently treated with RQ1 RNAse free DNAse (Promega, Madison, CA) and RNA quantified in a spectrophotometer nanodrop machine ND-1000 (Thermo Fisher Scientific, Wilmington, DE) at the Genomic facility at The Pennsylvania State University. First-strand cDNA was synthesized from 1 μg of total RNA using a Super Script III First-strand synthesis kit (Invitrogen, Carlsbad, CA) and oligo-dT primers, according to instructions described by the manufacturer, in a 20 μl reaction volume. Primers were checked for their efficiency using a standard curve for every 10-fold diluted cDNA and a single-peak amplification. RT-PCR was carried out in a Applied Biosystems 7300 Sequence Detection System at the Genomics Core Facility at The Pennsylvania State University, using the FastStart Universal SYBR Green Master (Rox)® with the following conditions: 2 min at 50 °C, 10 min at 95 °C, and 15 s at 95 °C for one cycle each, followed by 1 min at 60 °C, repeated for 40 cycles. In addition, a disassociation stage consisting of 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C was added. RT-PCRs (10 μl volume) contained: 2 μl of cDNA (10-times diluted), 1 μl of H_2O, and 1 μl of 10 nM forward and reverse primers, and 5 μl SYBR green master mix® on a triplicate run on 96 well plates. Each experiment consisted of three biological replicates, and the final averages of the threshold cycle (Ct) were used to calculate the changes on fold difference with the $2^{(-\Delta\Delta Ct)}$ where:

$$\text{Fold difference} = 2^{(-\Delta\Delta Ct)}$$

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$$

$$\Delta Ct_{sample} = Ct_{GOI} - Ct_{normalizer}$$

$$\Delta Ct_{calibrator} = Ct_{GOI} - Ct_{normalizer}$$

Table 3
Pathway, gene, NCBI accession number, *Brachypodium distachyon* gene identifier and forward and reverse real-time PCR primers used in this work.

Pathway	Gene	NCBI	*Brachypodium* gene[a]	Forward	Reverse
Housekeeping[b]	SAM-DC: S adenosyl methionine	DV482676	Bradi1g02580.1	TGCTAATCTGCTCCAATGGC	GACGCAGCTGACCACCTAGA
Peroxidases	POX2: Peroxidase	X85228	Bradi1g17850.1	TTCAGCAGCGCCTTTGCTT	CGAGTTAACCTTGGAGCAGACG
Ethylene	ACC: 1-aminocyclopropane-1-carboxylic acid	U35779	Bradi1g49966.1	TTGCATTGAGCCTGGATGGT	TGTGGCTATGTGTGACCCTCCT
Phenylpropanoid	4CL: 4-coumarate:CoA ligase	EU099350.1	Bradi3g37300.1	GTTCGAGACGGTGAGGATGTTC	CATACTTTCCAACAGCGTCGC
Jasmonic acid	COI1: Coronatine-insentive protein 1	HM447645.1	Bradi2g23730.2	AGTTCTCGCGGTGAGGAATGT	ATCATCATCCCCTCGCTCAAC
Jasmonic acid	LOX3: Lipoxygenase 3	HQ913602.1	Bradi1g11670.1	TCAACTTGCCCTTTCCACATG	GCAAACCGGATTAACTCCTGC
Salicylic acid	NPR1: Non-expressor of pathogenesis-related genes 1	AY323485.1	Bradi2g05870.1	CCTATTGAGGCAAGAGTGGCAA	GCCCGTGGTATCTCTGGAAGTT

[a] www.brachypodium.org.
[b] From Hong et al. [44].

RT-PCR data were first log-transformed, following the normality of the variances according to Snedecor and Cochran [45]. Results were then analysed as a randomized complete block design (RCBD), with three replicates and three different treatments (time-points) according to the experiment, using the PROC GLM of SAS Institute V.S. 9.2 [40]. Means were differentiated using the Least Significance Difference (LSD) protected of Fisher ($p \geq 0.05$) [41]. In addition, we used the REST 2009 (Relative Expression Software Tool) software, Quiagen® [46]. In this software, the relative expression of the gene is calculated as follows: "RE = Concentration of GOI/Concentration of N". Such software uses simple statistical randomization tests that allow the detection of the significances of the treated sample against the control sample [47]. In this experiment, the time-points after inoculation were considered as treatment samples while the samples without inoculations served as the control. More details in the mathematical model can be found in the user's guide for REST 2009 and the publications described herein [47].

3. Results and discussion

3.1. Screening B. distachyon genotypes

3.1.1. Growth performance of fall armyworm (FAW) larvae on B. distachyon genotypes

Caterpillars fed on maize and tall fescue served as controls for comparison with those on a *Brachypodium* diet. Their growth was excluded from the analysis of variance of the *Brachypodium* genotypes and a separate ANOVA was performed for caterpillars fed on maize and tall fescue.

After feeding for ten days on maize, lines Mp708, Tx601, and B73—previously reported to be resistant, medium susceptible, and highly susceptible to predation by insects—control FAW caterpillars reached an average weight of 317.5 ± 4.78, 345.2 ± 33.6, and 391.8 ± 32.64 mg, respectively. When fed on tall fescue, the caterpillars' average weight reached 322.2 ± 22.24 mg.

In the experimental feedings, FAW larval weights depended on which *Brachypodium* genotype they were fed ($P = 0.001$), as shown in Fig. 1. Larvae fed on genotypes CWA and CWC, in particular, gained more weight than larvae fed on the other *Brachypodium* genotypes; however, they still reached only about half the weight of larvae fed on resistant maize genotype Mp708. The very low performance of *Brachypodium* compared to maize indicates that *Brachypodium* would not be a preferred host for FAW, making this an unsuitable combination for further studies of insect–*Brachypodium* interaction. More work is necessary to determine which insects, if any, would be suitable to establish a model of *Brachypodium*–caterpillar feeding interactions. The possibility that FAW caterpillar growth was limited by specific metabolites produced by *Brachypodium*, and perhaps induced by the caterpillars' feeding, may be an area suitable for further studies.

3.1.2. Symptoms of Russian wheat aphid (RWA) attack on B. distachyon

The most noticeable symptom of the RWA attack on *B. distachyon* was leaf-chlorosis, with no evident effect on leaf-rolling; therefore, this parameter could not be evaluated. Evaluations on *Brachypodium* were made when the wheat variety Yuma, included as a control for susceptibility, reached the 8 or 9 rating score level (plants completely attacked) as previously described [48]. Highly significant differences ($P = 0.0001$) among *Brachypodium* genotypes were observed when *B. distachyon* was infested with RWA1, where the CWF genotype was the most resistant and CWC the most susceptible, with scores in the order of 1.0 and 6.5 on average, respectively (see Table 4). Biotype RWA2 was also found to be virulent ($P = 0.0001$) on *B. distachyon*, with the lowest score (3.4) recorded for CWF and the highest (8.0) for CWC (Table 4). Varied levels of resistance have been reported previously when different biotypes of RWA were tested with 24 wheat or barley cultivars [10]. The *Brachypodium* genotypes tested here were, in general, more resistant to biotype 1 compared to biotype 2, as reported previously, and Bd21 (CWB, in this study) showed increased resistance to RWA1 and susceptibility to RWA2, in agreement with Ref. [8]. In addition, our results also showed that *Bd* CWF was the most resistant genotype to both RWA biotypes, again in agreement with Ref. [8] study, which reported an inbred line developed from CWF (PI 245730) as the most resistant genotype to both RWA biotypes. The intermediate resistance for genotypes CWE and CWJ to RWA biotypes contrasts the previous report showing that inbred lines derived from CWE and CWJ were highly resistant [8]. These results indicate that the *Brachypodium*-RWA interaction could be useful to determine the gene(s) involved in the resistance to RWA and may also be suitable for determining QTLs associated with this interaction.

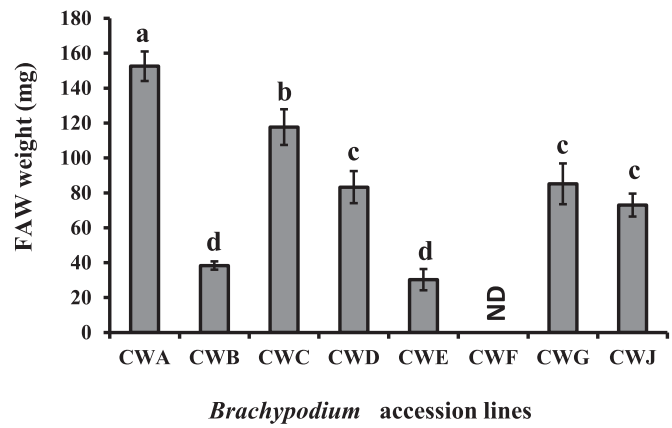

Fig. 1. FAW caterpillar mean weights after 10 days of continuous feeding with eight different *Brachypodium distachyon* accessions. Bars are mean ± SE ($N = 15$). Means followed by the same letter were not significantly different ($p \geq 0.05$) according to the Least Significant Difference (LSD) protected test of Fisher $p \geq 0.05$. ND = not determined.

3.1.3. Pathology of different fungal species on B. distachyon

M. grisea: Table 5 shows the lesion scores for the eight *B. distachyon* accessions one week after inoculation with five *M. grisea* isolates, formerly *Pirycularia orizae*, the causal agents of the Rice Blast disease. The rice cultivar Kitaake was used as a

Table 4
Mean chlorosis rating after two weeks of artificial infestation with Russian Wheat Aphid (*Diuraphis noxia*) biotypes RWA1 and RWA2 of eight different *Brachypodium distachyon* genotypes and wheat cultivar "Yuma" as control. Chlorosis was measured by visual rating on a scale of 1–10.

Genotype	*n*	RWA1	*n*	RWA2
CWA	8	1.9 ef	6	6.2 bc
CWB	9	5.6 c	9	5.8 bcd
CWC	8	6.5 b	8	8.0 ab
CWD	6	2.8 de	7	5.4 cde
CWE	8	2.3 de	7	4.0 cde
CWF	7	1.0 g	7	3.4 e
CWG	8	2.3 de	6	4.0 cde
CWJ	8	1.8 f	7	3.7 de
Yuma	7	8.4 a	8	8.9 a
LSD (0.05)		1.27		2.19

Means followed by the same letter within each column were not significantly different ($p \geq 0.05$) according to the Least Significant Difference (LSD) protected test of Fisher $p \geq 0.05$.

Table 5
Lesion type[a] in different *Brachypodium distachyon* genotypes after seven days of inoculation with the causal agent of Rice Blast disease, *Magnaporthe grisea* (Hebert) and Anthracnose, (*Colletotrichum cereale* Manns sensu lato Crouch, Clarke, and Hillman formerly *C. graminicola*) in grasses. Both pathogens cause foliar damage.

Genotype	*M. grisea*										*C. cereale*	
	n	4091-5-8	*n*	Guy 11	*n*	IE1K	*n*	18-1	*n*	IA45	*n*	246 CgMA-1
CWA	4	2.8 abc	4	3.0 ab	4	4.3 a	4	1.5 b	4	0.3 b	6	0.3 de
CWB	9	2.4 abc	4	3.0 ab	4	3.3 abc	4	1.0 b	4	0.3 b	6	1.2 cd
CWC	8	3.5 a	4	3.8 a	4	3.3 abc	4	3.0 a	4	0.3 b	6	2.0 bc
CWD	4	1.9 c	4	3.3 ab	3	2.8 cd	4	1.5 b	4	0.0 b	6	3.3 a
CWE	8	3.3 a	4	3.3 ab	4	2.0 de	4	1.0 b	4	0.3 b	6	1.0 de
CWF	5	1.7 c	4	1.0 c	3	3.0 bcd	4	0.0 c	4	0.0 b	6	0.0 e
CWG	5	1.9 bc	4	3.0 ab	4	3.0 bcd	4	1.5 b	4	1.5 a	6	2.7 ab
CWJ	5	2.4 abc	4	3.0 ab	4	4.0 ab	4	1.5 b	4	0.3 b	6	0.3 de
Kitaake	2	3.0 ab	2	2.0 bc	2	1.5 e	2	0.0 c	2	0.05 b		
LSD (0.05)		1.16		1.47		1.10		0.69		0.57		0.95

Means followed by the same letter within each column were not significantly different ($p \geq 0.05$) according to the Least Significant Difference (LSD) protected of Fisher $p \geq 0.05$.

[a] Scoring rating scale from 0 to 5, 0 = no visible evidence of infection to 5 = large eyespot lesion as used in rice [31] adapted to *B. distachyon* for *M. grisea*.

control because of its known resistance/susceptibility to different *M. grisea* isolates (see Table 5). Highly significant differences were found among *Brachypodium* genotypes for each of the five different isolates tested (ANOVAs not shown). The isolate 4091-5-8, which is a mix of two Japanese field isolates that infect weeping lovegrass and goosegrass, proved to be successful at infecting *B. distachyon* ($P = 0.0091$). Initiation of the disease symptoms was first observed 48 h post-inoculation, as shown for genotype CWA (Fig. 2e). The progression of these symptoms after 96 h is shown in Fig. 2f and after 120 h in Fig. 2g. Genotypes CWC and CWE showed the highest lesion score, reaching on average 3.5 and 3.3 respectively on a rating scale from 0 to 5 (Table 5). Symptoms on the leaves of genotypes CWC (susceptible) and CWD (resistant) one week after inoculation are shown in Fig. 2h and i, respectively.

The rice (*Oriza sativa*) isolate Guy 11, which comes from French Guyana, was also successful in producing disease symptoms among the eight *B. distachyon* genotypes ($P = 0.0199$), with the first symptoms visible four days after inoculation. Except for genotype CWF, which was highly resistant, all the other *Brachypodium* genotypes responded identically to attack by this isolate. Most of the *Brachypodium* genotypes ($P = 0.0031$) tested were heavily attacked by *M. grisea* isolate IE1K (Table 5). The aggressiveness of this isolate was observed two days after inoculation, at which point symptoms of the disease were clearly visible and the infection manifested as brown spots, which continued to grow. Highly significant differences were found between *Brachypodium* genotypes with isolate 18-1 ($P = 0.001$) and isolate IA45 ($P = 0.0007$); however, only slight symptoms of the disease were observed around five to six days after inoculation. A week after inoculation, no further progress was observed across most of the genotypes tested, except for genotype CWC, which scored 3.0 on the rating scale, and genotype CWF, which, in contrast, did not show any visible symptoms of the disease when inoculated with isolate 18-1.

Recently, *Brachypodium–M. grisea* has been proposed as a model for plant–fungus interaction studies using the isolate Guy 11, as its fungal development and disease symptomatology resembles that found in rice [4,49,50]. This study is the first attempt to investigate whether other *M. grisea* isolates, apart from Guy 11, could colonize *Brachypodium* and has shown that *Brachypodium*'s performance in fact varied according to the isolate. While isolates 4090-1-5-8 and Guy 11 clearly produced different levels of disease among *Brachypodium* genotypes, helping to discriminate between resistant and susceptible genotypes, these isolates mainly attack different hosts, with Guy 11 producing visible symptoms in rice and 4090-1-5-8 being more severe in lovegrass and goosegrass [37]. Furthermore, isolates 18-1 and IA-45, which produced very few disease symptoms in the *Brachypodium* genotypes tested, have been previously considered as moderately virulent in rice [51]; isolate IE1K, which was very aggressive in *Brachypodium*, has also been reported as highly virulent in rice [51]. CWF genotype was highly resistant against most of the *M. grisea* isolates tested. It is important to test a single isolate on many host genotypes to differentiate novel host–pathogen interactions. Concurrently, it is also important to test numerous isolates on a single host genotype.

C. cereale: Highly significant differences were observed among the different *Brachypodium* genotypes regarding foliar symptoms ($P = 0.001$) produced by *C. cereale* (Table 5). Genotypes CWD and CWG were most susceptible to *C. cereale*, and genotype CWF was the most resistant, showing no sign of the disease. In this case, leaf tissue only (not root tissue) was used for symptom evaluation, which started to be visible five days post-inoculation. Clearly, *C. cereale* isolate 246 CgMA-1 induced a foliar disease in *Brachypodium* similar to a foliar blight reported in *Poa annua*; however, it did not produce the known "basal rot" as previously reported in this species and in other grasses [13]. In addition, genotype CWF did not show any visible sign of disease, and although the disease developed in all of the other genotypes, none of the genotypes tested were completely colonized by this pathogen. As other isolates of *C. cereale* may or may not cause severe signs of the disease, it is highly recommended that more isolates be tested in order to determine the differences in resistance and susceptibility of *B. distachyon* to this pathogen.

S. homoeocarpa: We have tested two different isolates of the soil-borne pathogen *S. homoeocarpa*, which causes the dollar-spot disease in some grasses, for its ability to attack *B. distachyon*. Our results showed that *S. homoeocarpa* started to colonize *B. distachyon* two days after inoculation, with significant differences among the different *Brachypodium* inbred lines and PIs when inoculated with isolate S005044B ($P = 0.0001$). Evaluations made one week post-inoculation showed that CWA, CWF, CWG and CWJ were the most resistant (Fig. 2b), while the rest of the *Brachypodium* genotypes had an intermediate or relatively high level of susceptibility to isolate S005044B (Table 6). In contrast, the genotypes tested with isolate 241 ShMp-2 did not show any significant differences ($P = 0.4529$) in disease levels, all being intermediate in their levels of susceptibility (Table 6). This work demonstrates that *S. homoeocarpa* is able to colonize *B. distachyon*; further *Brachypodium–Sclerotinia* interaction studies should be useful to determine the gene(s) responsible for the resistance against this pathogen, making it possible to translate this information to other grass species. For instance, considering that the turfgrass industry reports billions of dollars of benefits, and approximately 70% of the fungicides applied in golf courses aim to control *S. homoeocarpa* and *R. solani* among other diseases [17], more breeding efforts need to be made in order to improve resistant varieties for these type of diseases. Turfgrass could benefit from information gained from *B. distachyon* as a model species.

P. aphanidermatum: The soil-borne pathogen *P. aphanidermatum*, isolate P3, also caused disease on *B. distachyon*. Although no significant differences were found among the majority of genotypes tested ($P = 0.0750$), in the final evaluation, one week post-inoculation, genotypes CWA, CWB, CWC, CWD, and CWJ showed similar levels of attack, and CWF showed hardly any symptoms at this time (Table 6). This situation might be different if more isolates were tested, as there was colonization of this pathogen on the *B. distachyon* plants. Although these differential interactions were weak, it may be important in the future to establish a more definitive *Brachypodium–Pythium* interaction, as this soil-borne

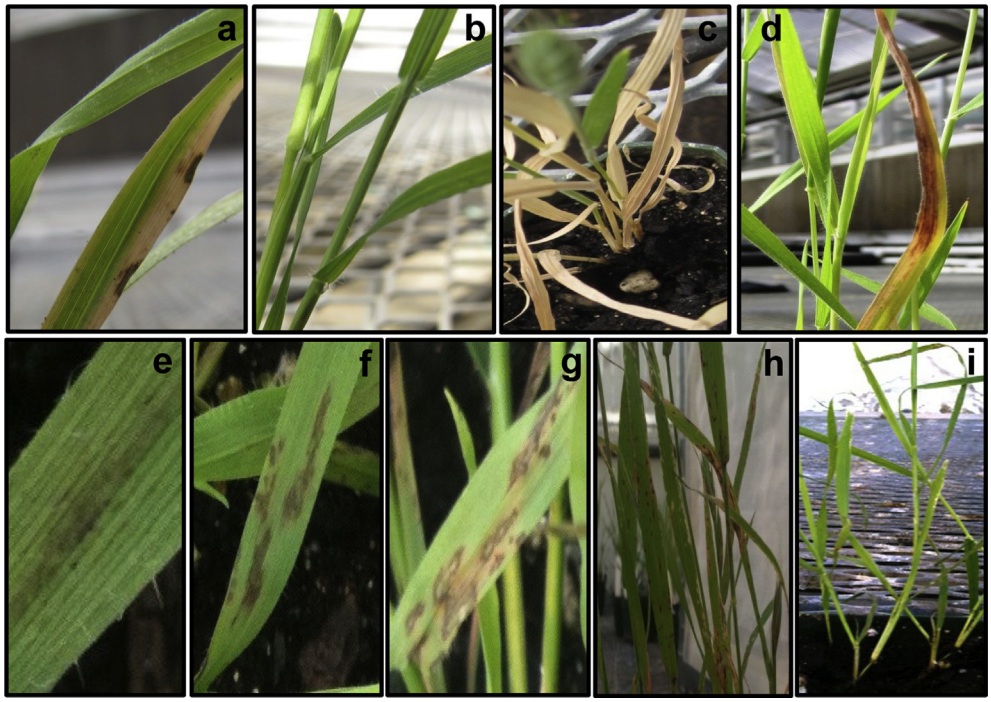

Fig. 2. Symptoms of *Sclerotinia homoeocarpa* (isolate S-00-5044B) infection on susceptible genotype CWC (a) and resistant genotype CWA (b) leaves 1 week after inoculation. Symptoms of *Ophiosphaerella agrostis* (isolate 81 OpMO4) infection on susceptible genotype CWC (c) and partially resistant genotype CWG (d) leaves 2 weeks after root inoculation; symptoms of *Magnaporthe grisea* (isolate 4090-1-5-8) infection on susceptible genotype CWA 48(e), 96 (f) and 120 (g) hours after inoculation and on genotypes CWC (h; partially resistant) and CWD (i; resistant) leaves one week after inoculation.

pathogen (among others) causes damping-off disease in grasses, which diminishes normal development and therefore crop yield.

R. solani: Isolate RhBC 2-1-3 of the soil-borne pathogen *R. solani* was used to test the eight *B. distachyon* genotypes for their resistance or susceptibility to this pathogen. Isolate RhBC 2-1-3 caused some symptoms on *Brachypodium* genotypes ($P = 0.001$) one week after inoculation, where genotypes CWC, CWD, and CWE showed the highest level of infection and genotypes CWF and CWJ did not show any signs of disease, even two weeks following inoculation (Table 6).

M. poae, *O. agrostis*, *O. korrae*, and *G. graminis* var. *avenae*: The soil-borne pathogens *M. poae*, *O. agrostis*, *O. korrae*, *G. graminis* var. *avenae*, which are important pathogens for the turf-grass industry, were evaluated for their pathogenesis against *B. distachyon*. Evaluations were made two to four weeks following inoculation, after which point the symptoms of disease showed no further progress. Highly significant differences were found among the *Brachypodium* lines ($P = 0.0001$) when infected with these pathogens. CWC was found to be the most susceptible to three of the four pathogens tested, reaching scores above 8.5, while for *O. korrae*, CWF was the most susceptible genotype (Table 7). Genotype CWF was also found to be resistant to *M. poae* and *G. graminis* var. *avenae*, genotype CWD to *O. korrae* and *G. graminis* var. *avenae*, and genotype CWG partially resistant (Fig. 2d) to *O. agrostis*. Fig. 2c shows symptoms of *O. agrostis* infection on susceptible genotype CWC, two weeks after root inoculation.

Table 6
Soil-borne pathogens scoring in different *Brachypodium distachyon* genotypes after 7 or 14 days following inoculation.

Genotypes	*Sclerotinia homoeocarpa*[a]				*Pythium*[a] *aphanidermatum*		*Rhizoctonia*[b] *solani*	
	n	S-00-5044B	*n*	241 ShMp-2	*n*	P3	*n*	RhBC 2-1-3
CWA	7	1.2 d	6	3.2 ab	7	2.1 ab	3	1.0 c
CWB	8	3.9 a	6	3.3 a	8	2.4 a	4	1.5 bc
CWC	7	3.1 ab	6	2.8 ab	8	2.5 a	4	3.0 a
CWD	7	2.3 bc	6	2.8 ab	8	2.4 a	4	2.8 a
CWE	5	3.0 ab	6	2.6 ab	8	1.8 ab	4	3.0 a
CWF	6	1.5 cd	5	2.7 ab	4	0.8 b	4	0.0 d
CWG	5	1.5 cd	6	2.7 ab	7	1.7 ab	4	1.8 b
CWJ	5	1.5 cd	5	2.2 b	2	2.5 a	4	0.0 d
LSD (0.05)		1.13		0.95		0.91		0.58

Means followed by the same letter within each column were not significantly different ($p \geq 0.05$) according to the Least Significant Difference (LSD) protected of Fisher $p \geq 0.05$.
[a] Results are shown for disease presence after 7 days post inoculation.
[b] Results are shown for disease presence after 14 days post inoculation.

Overall, our results have shown that, except for the FAW, the genotypes CWF (PI 245730), which was originally from Turkey, and CWG (PI 208216), from South Africa, were outstanding in terms of resistance to most of the fungi and insects tested. It has been shown previously that an inbred line derived from PI 245730 (CWF) was susceptible to the attack of *Puccinia graminis* in three different *formae speciales* (*f.s.*), which included *f.s. tritici*, *f.s. lolii*, and *f.s. phlei-pratensis* [7]. These contrasting results might be attributed to testing the PI itself and not the inbred. Heterogeneity has been previously reported in the *Brachypodium* collected accessions [52]; therefore, inbred lines were developed to overcome heterogeneity [3] or because PI 245730 does not possess resistance against *P. graminis*. However, we have showed that this PI was susceptible to *O. korrae*, reinforcing the need to establish plant–biotic stress interaction before starting functional studies of candidate genes for cultivated grasses.

3.2. Defense responses of Brachypodium to pathogen attack

The results from the pathogen/insects-screening experiments clearly showed significant susceptibility/resistance differences

Table 7
Scoring[a] for pathogens attacking the roots in different *Brachypodium distachyon* genotypes.

Genotype	*n*	*Magnaporthe poae* 626 MpNY-2	*n*	*Ophiosphaerella agrostis* 81 OpMO4	*n*	*Ophiosphaerella korrae* 548 OKCT-3	*n*	*Gaeumannomyces graminis* var. *avenae* 389 GGa CT-T
CWA	4	4.3 b	6	4.2 bc	3	5.3 b	4	4.8 c
CWB	3	2.5 cd	5	4.5 b	4	5.5 b	3	2.5 d
CWC	4	10.0 a	6	9.3 a	4	5.5 c	4	8.8 a
CWD	4	3.3 bc	6	2.3 bc	4	1.0 d	4	1.5 de
CWE	4	3.3 bc	6	4.3 bc	4	6.0 ab	3	4.5 c
CWF	4	1.0 d	6	2.3 bc	4	7.3 a	3	1.5 e
CWG	4	2.0 cd	6	1.8 c	4	2.3 c	4	1.3 e
CWJ	4	4.0 b	6	4.7 b	4	6.3 ab	3	7.0 b
LSD (0.05)		1.66		2.80		1.47		0.85

Means followed by the same letter within each column were not significantly different ($p \geq 0.05$) according to the Least Significant Difference (LSD) protected of Fisher $p \geq 0.05$.

[a] Evaluations were made according to the time of appearance of the sign and symptoms of the disease. *M. poae* a month after inoculation; *O. agrotis* three weeks after inoculation; *O. korrae* two weeks after inoculation; *G. graminis* two weeks after inoculation, on a scale from 1 to 10, where 1 = no symptoms and 10 = plant death.

amongst the *Brachypodium* genotypes tested, with CWF and CWC being overall the most resistant and most susceptible, respectively. In an attempt to elucidate the possible factors involved in these defense/resistance responses in *Brachypodium*, we determined the activity of genes known to be involved in the activation of defense-related pathways to pathogen attack in grasses such as rice, wheat, and maize, as well as the model plant *A. thaliana*. This included measurements of peroxidase enzyme activity and the expression of key genes of the jasmonic acid (JA), salicylic acid (SA), phenylpropanoid (PP), and ethylene (ET) pathways.

3.2.1. Induction of peroxidase enzyme activity in resistant and susceptible Brachypodium lines challenged with different pathogens

Pathogen-induced peroxidases have proved to be an effective defense response against both pathogen [27] and insect attack [28,29] and are well known for belonging to a large multigene family participating in a broad range of physiological processes, such as lignin and suberin formation, cross-linking of cell wall components, and metabolism of ROS and RNS [26].

The *Brachypodium* genotypes chosen for determining the levels of peroxidase (POX) activity following infection with different microorganisms were selected based on their response to the different pathogens and included the most-resistant and most-susceptible genotypes. In order to determine if POX is involved in the phenotypic resistance observed when *Brachypodium* lines were infected with the pathogens *S. homoeocarpa* and *O. agrostis*, peroxidases activity was investigated following infection of *Brachypodium* genotypes with *S. homoeocarpa* and *O. agrostis*. *Brachypodium* genotypes included the most-resistant and most-susceptible genotypes. Peroxidase activity was not investigated in the *Brachypodium*-insect interaction due to the poor response produced with the FAW and RWA. ANOVA showed no significant difference for POX activity, in either susceptible CWB ($P = 0.5219$) or resistant CWF ($P = 0.3811$) genotypes, at the different time points tested following inoculation with *S. homoeocarpa* isolate S-00-5044B (Fig. 3a). Similarly, POX activity in the susceptible genotype CWC and resistant genotype CWG to *O. agrostis* isolate 81 OpMO4 increased over 96 h following inoculation, with the increased activity in the resistant genotype being higher than in the susceptible one at each time point; however, differences among the different time points within each genotype were not significant (CWG, $P = 0.469$ and CWC, $P = 0.5116$) (Fig. 3b). Following inoculation with the foliar pathogen *M. grisea* isolate Guy 11 over 48 h, a non-replicated experiment showed that POX activity increased in resistant genotype CWF, reaching the highest level at 24 h after inoculation and a plateau in activity by 48 h, while POX activity in the susceptible genotype CWD showed little increase in activity (Fig. 3c).

When challenged with *C. cereale* isolate 246 CgMA-1, POX activity in resistance (CWF) and susceptibility (CWD) genotypes over 96 h following inoculation was found to be opposite to that expected: genotype CWD had a higher POX activity than genotype CWF. POX activity in CWD peaked 96 h after inoculation, while in CWF, POX activity peaked 72 h after inoculation (Fig. 3d). A similar result was found for POX activity in CWA and CWF genotypes when inoculated with the root-attacking pathogen *M. poae* isolate 626 MpNY-2 over 120 h following root inoculation, with POX activity being higher in the susceptible CWA genotype compared to the resistant CWF genotype (Fig. 3e).

Considering that little importance has been given to soil-borne plant/pathogen interaction in grasses, with limited studies in this field, we investigated possible resistance factors that may be involved in triggering responses in *Brachypodium* when infected with the soil-borne pathogens *S. homoeocarpa* and *O. agrostis* and preliminarily with *M. poae*, compared with the foliar pathogens *M. grisea* and *C. colletotrichum*. Similar to our results in *Brachypodium*, when infected with *S. homoeocarpa*, *C. cereale*, and *M. poae*, unexpectedly higher POX activity was also found in susceptible genotypes of wheat when inoculated with *Fusarium graminearum* [53] and in barley inoculated with *Erysiphe graminis* f.sp. *hordei* [54]. However, an increase in peroxidase activity has been reported in wheat when infected by *Puccinia triticina* [55], by *Pyricularia grisea* [56], or by *P. orizae* [57] in comparison with *Brachypodium* genotype CWF, which showed an increase in POX activity when infected with *M. grisea* (formerly *P. grisea*) (Fig. 3c). It should be stressed that POX activity levels reported in wheat following infection with different pathogens were much higher (over 100 mmol mg protein^{-1} min^{-1}) compared to values found in *Brachypodium*, as shown in Fig. 3.

Interestingly, 138 peroxidase genes have been found in rice, twice as many as in *A. thaliana* [16,27], and POX activity in rice has been shown to increase in resistant cultivars in response to fungal attack by pathogens such as *Bipolaris oryzae* [58] and *R. solani* [59]. Recently it has been reported that 106 of the POX genes in *B. distachyon* are not highly expressed [60], which might explain the low POX activity levels found in the pathosystems reported in the present study. Although POX may play an important role in defense mechanisms in grasses—and because a lack of correspondence between POX enzyme activity and pathogen resistance in both the dicot *A. thaliana* and in the monocot rice has been reported—these factors suggest that POX should be studied in greater detail in *B. distachyon*, as different interactions might lead to different responses that could be plant/pathogen specific.

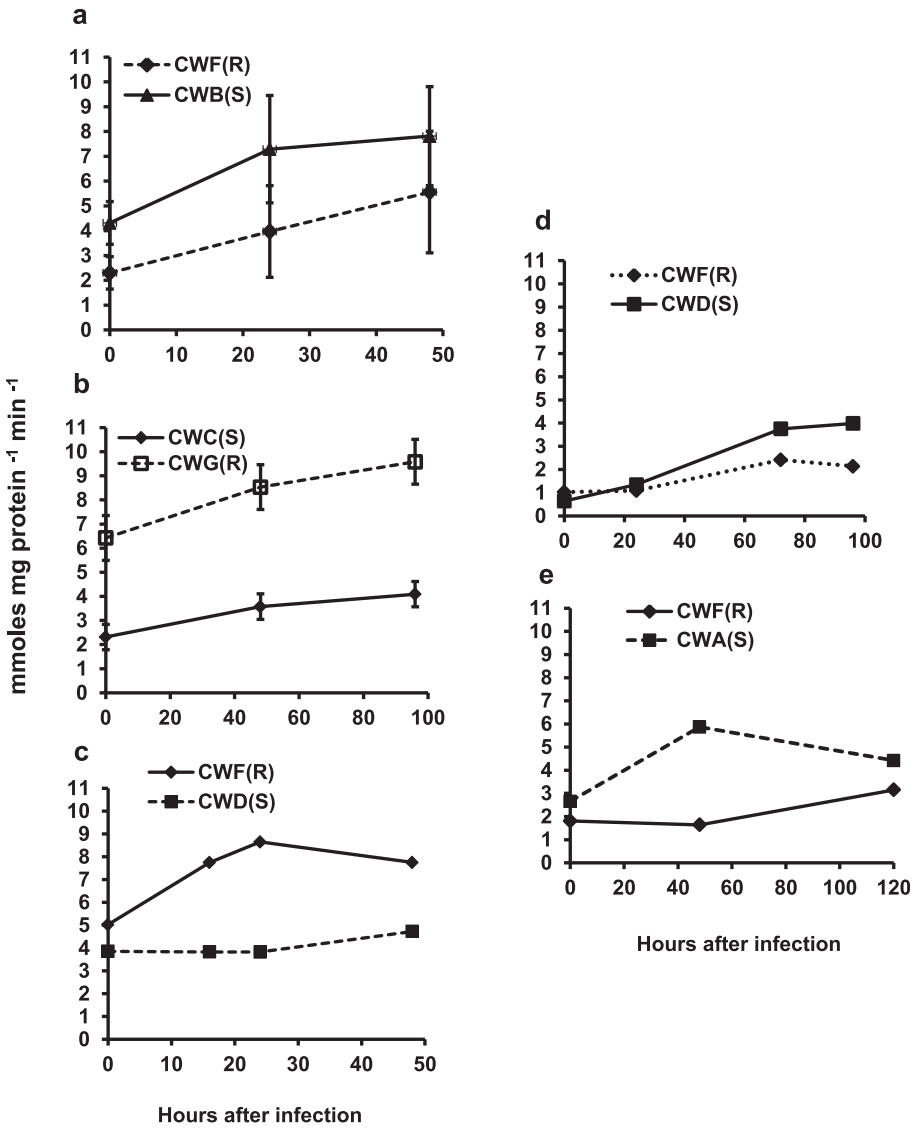

Fig. 3. Changes in peroxidase activity expressed as mmoles/mg protein/min in *B. distachyon* accessions (a) 48 h after inoculation of CWB(S) and CWF(R) with *Sclerotinia homoeocarpa* isolate S-00-544B; (b) 96 h after inoculation of CWC(S) and CWG(R) with *Ophiosphaerella agrostis* isolate 81 OpMO4; (c) 48 h after inoculation of CWD(S) and CWF(R) with *Magnaporthe grisea* isolate Guy 11; (d) 96 h after inoculation of CWD(S) and CWF(R) with *Colletotrichum cereale* isolate 246 CgMA-1; (e) 120 h after inoculation of CWA(S) and CWF(R) with *Magnaporthe poae* isolate MpNY-2.

3.2.2. Changes in the level of expression of key genes of defense-related pathways in resistant and susceptible Brachypodium genotypes challenged with different pathogens

The most significant differences in levels of resistance or susceptibility among the *Brachypodium* genotypes were found following infection with *S. homoeocarpa*. The transcript levels of some of the key genes likely involved in different pathogen-defense pathways were determined in order to understand the differences in disease resistance between these lines.

Aminocyclopropane-1-carboxylic acid (ACC). Although expression of ACC synthase, a key enzyme in the ethylene biosynthesis pathway, increased in the resistant genotype CWF 24 and 48 h post inoculation (hpi) with *S. homoeocarpa*, this was not statistically significant at ($P = 0.611$) and ($P = 0872$), respectively. The susceptible genotype CWB showed relatively small increases in expression of the ACC synthase at 24 hpi ($P = 0.848$) decreasing at 48 hpi ($P = 0.503$). Despite the fact that the changes observed between the different time-points were not significant within a genotype, the increase in ACC synthase expression shown here seems to indicate that the transcript levels for ACC synthase are higher in the resistant genotype CWF compared to susceptible genotype CWB (Fig. 4a).

Peroxidase (POX2). As indicated by increased mRNA levels determined by RT-PCR, the POX2 (Fig. 4b) and COI1 (not shown) genes did not show a significant change in expression in the resistant CWB genotype at either 24 hpi ($P = 0.79$ and $P = 0.972$ for POX2 and COI1, respectively) or at 48 hpi ($P = 0.119$ and $P = 0.582$ for POX2 and COI1, respectively). In CWB, POX2 transcript levels increased after 24 hpi with *S. homoeocarpa*, but not significantly ($P = 0.347$), and levels immediately decreased at 48 hpi ($P = 0.478$). As observed in Fig. 4b, the transcript levels of POX were higher in CWB compared to CWF.

4-Coumarate CoA ligase (4CL). The transcription levels for 4CL in the resistant CWF genotype showed no significant change after 24 h following inoculation with *S. homoeocarpa* (24 hpi $P = 0.564$)

(Fig. 4c). However, the dramatic decrease in expression levels after 48 hpi was highly significant ($P = 0.022$). Although some changes in 4CL expression have been shown in the CWB genotype, along with the time-points studied following inoculation with *S. homoeocarpa*, no significant changes were detected either at 24 hpi ($P = 0.327$) or 48 hpi ($P = 0.759$) (Fig. 4c). Following inoculation with *O. agrotis* isolate OpMO4, the transcription levels of 4CL in the resistant genotype CWG increased after 48 hpi ($P = 0.360$), being significantly higher at 96 hpi ($P = 0.04$). Following inoculation with *O. agrostis*, the increased expression in levels of 4CL in the resistant genotype CWG contrasted with the down-regulation in expression in the susceptible genotype CWC at 96 hpi ($P = 0.027$) (Fig. 4f). We have also observed a down-regulation of the expression of 4CL in the resistant genotype CWF when infected with *M. grisea* isolate 4091-5-8, even though the transcript levels for this gene were not significantly high ($P = 0.849$ at 24 hpi and $P = 0.748$ at 48 hpi) (data not shown).

Lipoxygenase 3 (LOX3). The transcription levels of the LOX3 gene in the JA pathway studied here were up-regulated in the resistant CWF genotype at 24 hpi ($P = 0.703$) with a decrease observed at 48 hpi ($P = 0.810$) following inoculation with *S. homoeocarpa* isolate S-00-5044B. Following inoculation, changes in LOX3 expression at the different time-points were barely observed in CWB ($P = 0.828$ at 24 hpi and $P = 0.622$ at 48 hpi) (Fig. 4d). We have also observed a down-regulation of the expression of LOX3 at 48 hpi ($P = 0.516$), which was significantly up-regulated at 96 hpi ($P = 0.005$) in the resistant genotype CWG following inoculation with *O. agrotis* isolate OpMO4 (data not shown).

Non-expressor of pathogenesis-related genes 1 (NPR1). While the NPR1 gene of the SA pathway was significantly suppressed at 48 hpi ($P = 0.0001$) in the resistant genotype CWF, its expression in CWB increased at 24 hpi, but not significantly ($P = 0.828$), and decreased at 48 hpi ($P = 0.486$) (Fig. 4e).

It has been shown that ethylene and jasmonic acid can act synergistically or antagonistically in response to stresses, and the relationship between these two pathways has been demonstrated genetically to be due to the transcription factor of ERF1 (Ethylene Response Factor 1) [61]. It is not surprising that plants, immediately after infection, can deploy a series of defenses such as those elicited by JA and ET dependent genes. Our results also show the increase in expression of 4CL following inoculation with *S. homoeocarpa*, which in combination with other enzymes is required for the activation of the phenylpropanoid pathway [62] and has been reported to be involved in the resistance of plants against pathogens [63]. It is also evident that fungal endo-xylanases can trigger pathogen responses [64,65], and expression of a *Trichoderma reesei* endo-xylanase in tall fescue under a senescence promoter targeted to the apoplast has been shown to trigger plant defense responses analogous to pathogen attack, leading to increased cell wall ferulate dimerization—a response that is likely to be mediated by ethylene and H_2O_2 [66]. This is consistent with results when infected *Brachypodium* plants showed an increase in expression levels of ACC synthase following inoculation with *S. homoeocarpa* and *O. agrostis*, pathogens known to secrete xylanase. Peroxidases gene expression showed the same response pattern as LOX3 and COI1 in both the resistant (CWF) and susceptible (CWB) genotypes, namely and respectively with an increase in expression at 24 h and a decrease at 48 h following infestation with *S. homoeocarpa*. However, when the relative levels of expression are compared between CWF and CWB (Fig. 4b), the susceptible genotype CWB had a higher expression level of POX in agreement with its higher level of enzyme activity (Fig. 3a).

Salicylic acid (SA) has proved to play a role in activation of plant defense against pathogens [25], with (NPR1) as the key transducer for activation of systemic acquired resistance (SAR) by SA. In this

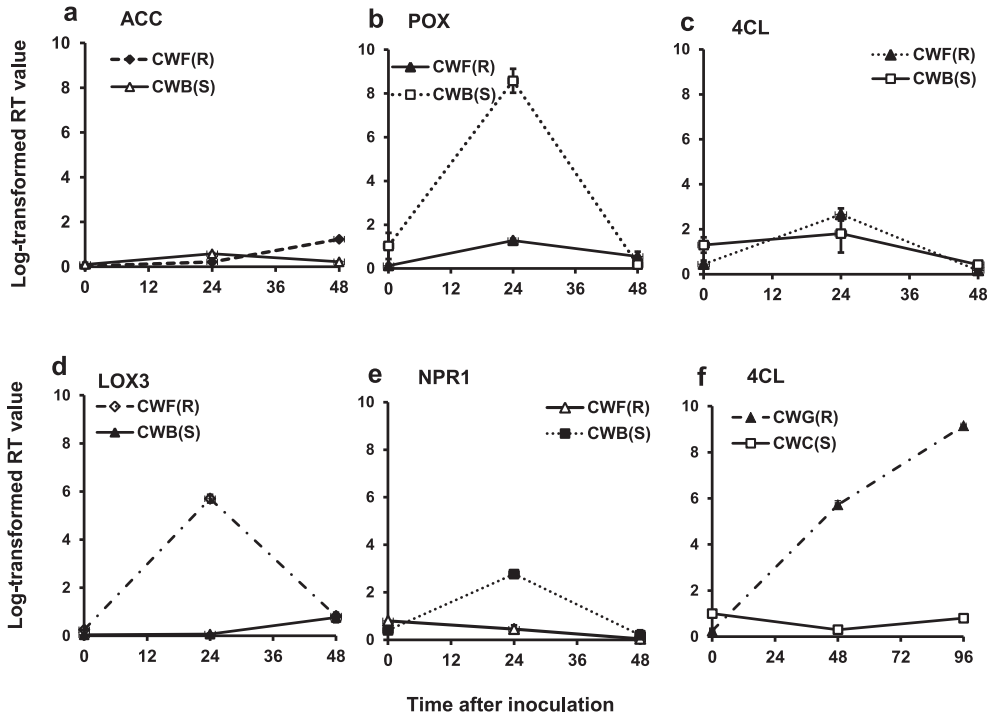

Fig. 4. Log-transformed relative quantification values (RT) of expression of genes involved in different pathogen defence pathways for a resistant (CWF) and a susceptible (CWB) *B. distachyon* accession over 48 h following inoculation with *S. homoeocarpa* isolate S-00-5044B. (a) ACC: 1-aminocyclopropane-1-carboxylic acid synthase for the ethylene pathway; (b) POX: peroxidase; (c) 4CL: 4-coumarate:CoA ligase for the phenylpropanoid pathway; (d) LOX3: lipoxygenase 3 and (e) NPR1: non-expressor of pathogenesis-related genes 1 for salicylic acid pathway and (f) for a resistant (CWG) and a susceptible (CWC) *Brachypodium* accession over 96 h following inoculation with *Ophiosphaerella agrostis* isolate 81 OpMO4, for 4CL for the phenylpropanoid pathway. The number of *n* is equal to 3 replicates; each replicate consisted of 3 biological replicates.

work, this gene was down-regulated when CWF was infected with *S. homoeocarpa*. Taking into account that *S. homoeocarpa* is a necrotrophic pathogen and that the hormone that seems to be activated is JA and/or ET, when *Brachypodium* lines are infected with this type of pathogen, it is not surprising that NPR1 was down-regulated, as it has been shown that the activation of one hormone, whether SA or JA, can suppress the action of the other in *A. thaliana* [30,31]. At this point we do not have enough data to determine if the synergism between JA and ET could happen in *B. distachyon* as in *A. thaliana* [64], but to study this synergism further as a possible defense mechanism could prove to be very interesting.

Inoculation of the roots of resistant genotype CWG with *O. agrotis* isolate OpMO4 resulted in an increase in expression of 4CL, as shown by the increase in its transcription levels, in the leaf tissue at 48 hpi ($P = 0.360$), being significantly higher at 96 hpi ($P = 0.04$). The increased expression in levels of 4CL in the resistant genotype CWG following infection with *O. agrostis* contrasted with the down-regulation in expression in the susceptible genotype CWC at 96 hpi ($P = 0.027$) (Fig. 4f). In contrast, we have observed a suppression of the expression of LOX3 (down-regulation) at 48 hpi ($P = 0.516$), which was significantly up-regulated at 96 hpi ($P = 0.005$) (data not shown). A similar trend was observed for 4CL expression in the leaves of resistant genotype CWF when infected with *M. grisea* isolate 4091-5-8, even though the transcript levels for this gene were not significantly high ($P = 0.849$ at 24 hpi and $P = 0.748$ at 48 hpi) (data not shown).

It has been demonstrated that resistance against pathogens can be attributed to multiple factors, and this may be the case in the resistance of genotype CWG when the roots were infected with *O. agrostis* and in genotype CWF when infected with *M. grisea*. In contrast to what was previously found with *S. homoeocarpa*, POX activities in the resistant genotypes CWG and CWF were higher when compared to the susceptible lines CWC and CWD. In addition, only 4CL seemed to be activated when *Brachypodium* was inoculated with *O. agrostis* and *M. grisea*. Like phenylalanine ammonia-lyase (PAL) and cinnamate-4-hydroxylase (C4H), 4CL enzyme is also required for the biosynthesis of compounds generated through the phenylpropanoid pathway, such as flavonoids and lignin [62]. As such, further studies are necessary to better understand processes such as the accumulation of flavonoids and lignin deposition, among others, that may be happening in the model grass in response to the attack of different pathogens. The high expression found for one of the genes responsible for the phenylpropanoid pathway in this work is opening the opportunity to investigate such specific topics using the interactions proposed in this investigation.

In conclusion, establishing such plant–pathogen interactions in *Brachypodium* as an alternative host will be helpful to understand the factors responsible for plant resistance/susceptibility and in developing better control regimes for pests and diseases attacking cultivated grasses. Significantly faster developments are more likely to be made with *Brachypodium* as compared to other grasses, as it is inbreeding, easy to grow, has a short life cycle and a small genome, and highly inbred lines are available for study—factors that can significantly accelerate research programs. The fungi described here, such as *S. homoeocarpa*, *O agrostis*, *R. solani* and *O. korrae,* which were able to colonize *B. distachyon* and which are relevant pathogens of the turfgrass industry as well of other cultivated grasses, could serve well for those purposes. The increased interest in grasses for biofuel production during recent years is also likely to lead to increasing levels of biotic stresses, as these species could be subjected to monoculture conditions [14,67]. In addition, increased efforts are being put into modifying the cell wall composition of grasses in order to increase their degradability and reduce the costs involved in ethanol production. Such attempts to modify lignin and cellulose [67], or to decrease cell wall ferulate cross-links [66,68], may well have significant impacts on their pathology. An example of this is the demonstration that reducing the level of cell wall ferulate cross-linking in tall fescue (*F. arundinacea*) by expressing an *Aspergillus niger* ferulic acid esterase had a positive effect on all measures of armyworm larval performance examined and demonstrated that susceptibility to insect herbivores could be increased when the level of ferulate cross-linking in grass cell walls was reduced [69].

Acknowledgements

The authors acknowledge the National Research Initiative on Agriculture and Food Research Initiative Competitive Grants Program grant No. 2008-02863 from the USDA National Institute of Food and Agriculture, for funding. Drs. John Kaminski, Wakar Uddin and Yinong Yang at The Pennsylvania State University (PSU) for supplying the pathogens. Dr. Dawn Luthe at PSU for providing the FAW and Dr. Gary Puterka at Plant Science Research Laboratory, USDA-ARS Stillwater, OK, for supplying the RWA (aphids). Vicki L. Bradley, USDA, ARS, WRPIS, Washington State University for supplying *Brachypodium distachyon* seeds. Dr. Mandeep Sharma for running some of the RT-PCRs and Dr. Phil Morris for critically reading the manuscript.

References

[1] Huala E, Dickerman AW, Garcia-Hernandez M, Weems D, Reiser L, LaFond F, et al. The *Arabidopsis* information resource (TAIR): a comprehensive database and web-based information retrieval, analysis, and visualization system for a model plant. Nucl Acids Res 2001;29:102–5.
[2] The International Brachypodium Initiative. Genome sequencing and analysis of the model grass *Brachypodium distachyon*. Nature 2010;463:763–8.
[3] Vogel JP, Garvin DF, Leong OM, Hayden DM. *Agrobacterium*-mediated transformation and inbred line development in the model grass *Brachypodium distachyon*. Plant Cell, Tissue Organ Cult 2006;84:199–211.
[4] Parker DM, Beckmann DP, Enot DP, Overy Z, Rios ZC, Gilbert M, et al. Rice blast infection of *Brachypodium distachyon* as a model system to study dynamic host/pathogen interactions. Nat Prot 2008;3:435–45.
[5] Peraldi A, Baccari G, Steed A, Nicholson P. *Brachypodium distachyon*: a new pathosystem to study *Fusarium* head blight and other *Fusarium* diseases of wheat. BMC Plant Biol 2011;1:100.
[6] Ayliffe M, Singh D, Park R, Moscou M, Pryor T. Infection of *Brachypodium distachyon* with selected grass rust pathogens. Mol Plant–Microbe Interact 2013;26:946–57.
[7] Figueroa M, Alderman S, Garvin DF, Pfender WF. Infection of *Brachypodium distachyon* by formae speciales of *Puccinia graminis*: early infection events and host pathogen incompatibility. PLoS ONE 2013;8(2):e56857. http://dx.doi.org/10.1371/journal.pone.0056857.
[8] Azhaguvel P, Li W, Rudd JC, Gill BS, Michels Jr JG, Wen Y. Aphid feeding response and microsatellite-based genetic diversity among diploid *Brachypodium distachyon* (L) Beauv accessions. Plant Gen Res: Charact Util 2008;7:72–9.
[9] Ashley TR, Wiseman BR, Davis FM, Andrews KL. The fall armyworm: a bibliography. Fl Entomol 1989;72:152–200.
[10] Randolph TL, Peairs F, Weiland A, Rudolph JB, Puterka G. Plant responses to seven Russian wheat aphid (Hemiptera: Aphididae) biotypes found in the United States. J Econ Entomol 2009;102:1954–9.
[11] Howard RJ, Valent B. Breaking and entering: host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. Annu Rev Microbiol 1996;50:491–512.
[12] Talbot NJ. On the trail of a cereal killer: exploring the biology of *Magnaporthe grisea*. Annu Rev Microbiol 2003;57:177–202.
[13] Inguagiato J, Murphy JA, Clarke BB. Anthracnose severity on annual bluegrass influenced by nitrogen fertilization, growth, regulators, and verticutting. Crop Sci 2008;48:1595–607.
[14] Crouch JA, Beirn LA, Cortese LM, Bonos SA, Clarke BB. Anthracnose disease of switchgrass caused by the novel fungal species *Colletotrichum navitas*. Mycol Res 2009;113:1411–21.
[15] Nelson EB, Craft CM. Identification and comparative pathogenicity of *Pythium* spp. from roots and crowns of turfgrasses exhibiting symptoms of root rot. Phytopathology 1991;81:1529–36.
[16] Settle DM, Fry JD, Tisserat NA. Development of brown patch and *Pythium* blight in tall fescue as affected by irrigation frequency, clipping removal, and fungicide application. Plant Dis 2001;85:543–6.
[17] Bonos SA, Casler MD, Meyer WA. Inheritance of dollar spot resistance in creeping bentgrass. Crop Sci 2003;43:2189–96.

[18] Kaminski JE, Dernoeden PH, O'Neill NR, Momen B. Reactivation of bentgrass dead spot and growth, pseudothecia production, and ascospore germination of *Ophiosphaerella agrostis*. Plant Dis 2002;86:1290–6.
[19] Bithell SH, Buttler RC, Harrow S, McKay A, Cromey MG. Susceptibility to take-all of cereal and grass species, and their effects of pathogen inoculums. Ann Appl Biol 2011;159:252–66.
[20] Tredway LP. Genetic relationships among *Magnaporthe poae* isolates from turfgrass hosts and relative susceptibility of 'penncross' and 'penn-A4' creeping bentgrass. Plant Dis 2006;90:1531–8.
[21] Maffei ME, Mithöfer A, Boland W. Insects feeding on plants: rapid signals and responses preceding the induction of phytochemical release. Phytochemistry 2007;68:2946–59.
[22] Furman-Matarasso N, Cohen E, Du Q, Chejanovsky N, Hanania U, Avni A. A point mutation in the ethylene-inducing xylanase elicitor inhibits the β-1-4-endoxylanase activity but not the elicitation activity. Plant Physiol 1999;121:345–51.
[23] Vanacker H, Carver TLW, Foyer CH. Early H_2O_2 accumulation in mesophyll cells leads to induction of Glutathione during hyper-sensitive response in the barley–powdery mildew interaction. Plant Physiol 2000;123:1289–300.
[24] Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, et al. A central role of salicylic acid in plant disease resistance. Science 1994;266:1247–50.
[25] Mou Z, Fan W, Dong X. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell 2003;113:935–44.
[26] Almagro L, Gómez Ros LV, Belchi-Navarro S, Bru R, Ros Barceló A, Pedreño MA. Class III peroxidases in plant defense reactions. J Exp Bot 2009;60:377–90.
[27] Passardi F, Longet D, Penel C, Dunand C. The class III peroxidase multigenic family in rice and its evolution in land plants. Phytochemistry 2004;65:1879–93.
[28] Felton G, Donato K, Del Vecchio R, Duffey S. Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. J Chem Ecol 1989;15:2667–94.
[29] Duffey S, Felton G. Enzymatic antinutritive defenses of the tomato plant against insects. In: Hedin P, editor. Naturally occurring pest bioregulators. Washington, D.C.: Amer Chem Soc; 1991. pp. 166–97.
[30] Spoel SH, Johnson JS, Dong X. Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. Proc Natl Acad Sci U S A 2007;104:18842–7.
[31] Spoel SH, Koornneef A, Claessens SM, Korzelius JP, Van Pelt JA, Mueller MJ, et al. NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways trough a novel function in the cytosol. Plant Cell 2003;15:760–70.
[32] Chippendale GM. Metamorphic changes in fat body proteins of the south-western corn borer, *Diatraea grandiosella*. J Insect Physiol 1970;16:1057–68.
[33] Peiffer M, Felton GW. Do caterpillars secrete "oral secretions"? J Chem Ecol 2009;35:326–35.
[34] Williams WP, Davis FM, Windham GL. Registration of Mp708 germplasm line of maize. Crop Sci 1990;30:757.
[35] Webster JA, Starks KJ, Burton RL. Plant resistance studies with *Diuraphis noxia* (Homoptera: Aphididae), a new United States wheat pest. J Econ Entomol 1987;80:944–9.
[36] Burd JD, Burton RL, Webster JA. Evaluation of Russian wheat aphid (Homoptera: Aphididae) damage on resistant and susceptible hosts with comparisons of damage ratings to quantitative plant measurements. J Econ Entomol 1993;86:974–80.
[37] Valent B, Farrall L, Chumley FG. *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. Genetics 1991;127:87–101.
[38] Cardwell KF, Happerly PR, Frederiksen RA. Pathotypes of *Colletotrichum graminicola* and seed transmission of sorghum anthracnose. Plant Dis 1989;73:255–7.
[39] Park DS, Sayler RJ, Hong Y-G, Nam M-H, Yang Y. A method for inoculation and evaluation of rice sheath blight disease. Plant Dis 2008;92:25–9.
[40] SAS. The SAS system SAS online doc HTML format version 9.2. Cary, North Carolina: SAS Institute Inc.; 2010.
[41] Steel RGD, Torrie JH. Principles and procedures of statistic in biological research. 2nd ed. New York, New York: McGraw-Hill; 1980.
[42] Ridge I, Osborne DP. Hydroxyproline and peroxidases in cell wall of *Pisum sativum*: regulation by ethylene. J Exp Bot 1970;21:843–56.
[43] Bradford MM. A dye binding assay for protein. Anal Biochem 1976;72:248–54.
[44] Hong S, Seo PJ, Yang MS, Xiang F, Park CM. Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR. BMC Plant Biol 2008;8:112.
[45] Snedecor GW, Cochran WG. One-way classification: the logarithmic transformation. In: Snedecor GW, Cochran WG, editors. Statistical methods. 6th ed. Ames, IA: Iowa State University Press; 1972. pp. 329–30.
[46] http://www.gene-quantification.de/REST_2009_Software_User_Guide.pdf.
[47] Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucl Acids Res; 2002:30–6.
[48] Haley SD, Peairs FB, Walker CB, Rudolph JB, Randolph TL. Occurrence of a new Russian wheat aphid biotype in Colorado. Crop Sci 2004;44:1589–92.
[49] Draper J, Mur LAJ, Jenkins G, Ghosh-Biswas GC, Bablak P, Hasterok R, et al. *Brachypodium distachyon*. A new model system for functional genomics in grasses. Plant Physiol 2001;127:1539–55.
[50] Routledge APM, Shelley G, Smith JV, Talbot NJ, Draper J, Mur LAJ. *Magnaporthe grisea* interactions with the model grass *Brachypodium distachyon* closely resemble those with rice (*Oryza sativa* L. Mol Plant Pathol 2004;5:253–65.
[51] Helliwell E, Wang Q, Yang Y. Transgenic rice with inducible ethylene production exhibits broad-spectrum disease resistance to the fungal pathogens *Magnaporthe oryzae* and *Rizhoctonia solani*. Plant Biotechnol J 2013;11:33–42.
[52] Christiansen P, Didion T, Andersen CH, Folling M, Nielsen KK. A rapid and efficient transformation protocol for the grass *Brachypodium distachyon*. Plant Cell Rep 2005;23:751–8.
[53] Mohammadi M, Kazemi H. Changes in peroxidase and polyphenol oxidase activities in susceptible and resistant wheat heads inoculated with *Fusarium graminearum* and induced resistance. Plant Sci 2002;162:491–8.
[54] Kery K, Somerville S. Enhancement of specific intercellular peroxidases following inoculation of barley with *Erysiph graminis* f.sp. *hordei*. Physiol Mol Plant Pathol 1989;35:323–37.
[55] Cawood ME, Pretorious JC, Van der Westhuizen AJ, Pretorious ZA. Disease development and PR-protein activity in wheat (*Triticum aestivum*) seedlings treated with plant extracts prior to leaf rust (*Puccinia triticina*) infection. Crop Prot 2010;29:1311–9.
[56] Xavier-Filha MS, Rodrigues FA, Dominiciano GP, Oliveira HV, Silveira PR, Moreira WR. Wheat resistance to leaf blast mediated by silicon. Austral Plant Pathol 2011;40:28–38.
[57] Debona D, Rodrigues FÁ, Rios JA, Nascimento KJT. Biochemical changes in the leaves of wheat plants infected by *Pyricularia oryzae*. Phytopathology 2012;102:1121–9.
[58] Silva MRJ, Pereira SC, Rodrigues FA, Zanão-Júnior LA, Fontes RLF, Oliveira MGA. Silicon and manganese on the activity of enzymes involved in rice resistance against brown spot. Trop Plant Pathol 2012;37:339–45.
[59] Saikia R, Kumar R, Arora DK, Gogoi DK, Azad P. *Pseudomonas aeruginosa* inducing rice resistance against *Rhizoctonia solani*: production of salicylic acid and peroxidases. Folia Microbiol 2006;51(5):375–80.
[60] Rawal HC, Singh NK, Sharma TR. Conservation, divergence, and genome-wide distribution of PAL and POX A gene families in plants. Int J Genom; 2013. Article ID 678969, 10p.
[61] Lorenzo O, Piqueras R, Sanchez-Serrano JJ, Solano R. Ethylene Response Factor1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell 2003;15:165–78.
[62] Lee D, Ellard M, Wanner LA, Davis KR, Douglas CJ. The *Arabidopsis thaliana* 4-coumarate:Co ligase (4CL) gene: stress and developmentally regulated expression and nucleotide sequence of its DNA. Plant Mol Biol 1995;5:871–4.
[63] Hahlbrock K, Scheel D. Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol 1989;40:347–69.
[64] Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. Plant J 1997;11:1187–94.
[65] Hückelhoven R, Dechert C, Kogel K-H. Non-host resistance of barley is associated with a hydrogen peroxide burst at sites of attempted penetration by wheat powdery mildew fungus. Mol Plant Pathol 2001;2:199–205.
[66] Buanafina MMdeO, Langdon T, Hauck B, Dalton S, Timms-Tavarella E, Morris P. Targeting expression of a fungal ferulic acid esterase to the apoplast, endoplasmic reticulum or Golgi can disrupt feruloylation of the growing cell wall and increase the biodegradability of tall fescue (*Festuca arundinacea*). Plant Biotechnol J 2010;8:316–31.
[67] Li X, Weng JK, Chapple C. Improvement of biomass trough lignin modification. Plant J 2008;54:569–81.
[68] Buanafina MMdeO, Langdon T, Hauck B, Dalton S, Morris P. Expression of a fungal ferulic acid esterase increases cell wall digestibility of tall fescue (*Festuca arundinacea*). Plant Biotechnol J 2008;6:264–80.
[69] Buanafina MMdeO, Fescemyer HW. Modification of esterified cell wall phenolics increases vulnerability of tall fescue to insect herbivory by the fall armyworm. Planta 2012;236:513–23.