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Specific peroxidases differentiate *Brachypodium distachyon* accessions and are associated with drought tolerance traits

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• **Background and Aims** *Brachypodium distachyon* (*Brachypodium*) is a model system for studying cereal, bioenergy, forage and turf grasses. The genetic and evolutionary basis of the adaptation of this wild grass species to drought stress is largely unknown. Peroxidase (POD) may play a role in plant drought tolerance, but whether the allelic variations of genes encoding the specific POD isoenzymes are associated with plant response to drought stress is not well understood. The objectives of this study were to examine natural variation of POD isoenzyme patterns, to identify nucleotide diversity of *POD* genes and to relate the allelic variation of genes to drought tolerance traits of diverse *Brachypodium* accessions.

• **Methods** Whole-plant drought tolerance and POD activity were examined in contrasting ecotypes. Non-denaturing PAGE and liquid chromatography–mass spectrometry were performed to detect distinct isozymes of POD in 34 accessions. Single nucleotide polymorphisms (SNPs) were identified by comparing DNA sequences of these accessions. Associations of *POD* genes encoding specific POD isoenzymes with drought tolerance traits were analysed using TASSEL software.

• **Key Results** Variations of POD isoenzymes were found among accessions with contrasting drought tolerance, while the most tolerant and susceptible accessions each had their own unique POD isoenzyme band. Eight POD genes were identified and a total of 90 SNPs were found among these genes across 34 accessions. After controlling population structure, significant associations of *Bradi3g41340.1* and *Bradi1g26870.1* with leaf water content or leaf wilting were identified.

• **Conclusions** *Brachypodium* ecotypes have distinct specific POD isozymes. This may contribute to natural variations of drought tolerance of this species. The role of specific *POD* genes in differentiating *Brachypodium* accessions with contrasting drought tolerance could be associated with the general fitness of *Brachypodium* during evolution.

Key words: *Brachypodium distachyon*, drought tolerance, gene and trait association, natural variation, peroxidase, single nucleotide polymorphism.

INTRODUCTION

Brachypodium distachyon (*Brachypodium*) is a temperate, monocot wild grass species and it possesses all the qualities to make it an excellent model organism (Garvin *et al*., 2008; Bevan *et al*., 2010). It has diploid ecotypes containing five chromosomes, easily distinguishable chromosomes ($2n = 10$), small genome size (approx. 300 Mbp), self-fertility, small physical status, short life cycle and a simple growth requirement (Draper *et al*., 2001). This species is phylogenetically closer to some economically important food and bioenergy crops than is rice (*Oryza sativa*) (Draper *et al*., 2001), thus providing a powerful tool for studying functional and ecological genomics aimed at improving grain, forage and bioenergy crops. To date, genetic and genomic resources for *Brachypodium* have been developed, including the entire genomic sequence (Vogel *et al*., 2010), a genetic linkage map (Garvin *et al*., 2010), a high-efficiency transformation system (Vogel *et al*., 2006; Pãcurar *et al*., 2008; Vogel and Hill, 2008), T-DNA mutants (Bragg *et al*., 2012; Thole *et al*., 2012) and genome diversity (Gordon *et al*., 2014). All these tools and resources are valuable for examining the genetic and evolutionary basis of complex traits such as abiotic stress tolerance.

Antioxidant metabolism plays a role in drought tolerance of the plants. As one of the reactive oxygen species, hydrogen peroxide (H_2O_2) is a by-product of biological reactions in the plant cell. Adverse environmental conditions such as drought stress can promote excess accumulation of H_2O_2, potentially leading to oxidative damage to protein, DNA and lipids (Apel and Hirt, 2004). The degree of lipid peroxidation, indicated by the level of malondialdehyde (MDA) content, often increased under drought stress in plant species (Zhang and Kirkham, 1996; Jiang *et al*., 2010; Xu *et al*., 2011; Liu and Jiang, 2015). Removal of H_2O_2 is accomplished by the action of several antioxidant enzymes at different cellular locations. Of the antioxidative enzymes, peroxidase (POD) catalyses the reduction of H_2O_2 to water, followed by subsequent oxidation of small molecules (Smith and Veitch, 1998). Drought stress often induces POD

activity in many plant species (Jung, 2004; Sofo *et al*., 2005; Upadhyaya *et al*., 2008; Selote and Khanna-Chopra, 2010; Ying *et al*., 2015). The tolerant genotype of maize (*Zea mays*) exhibited lower accumulation of MDA and H_2O_2 content related to increasing activities of POD and other antioxidant enzymes under water stress conditions (Moussa and Abdel-Aziz, 2008). Increases in POD activities were also found at the vegetative and flowering stages of seven species within the genus *Avena* under drought stress, coupled with an increased level of lipid peroxidation (Pandey *et al*., 2010). However, POD activity also remained unchanged in some annual and perennial grass species exposed to drought stress (Zhang and Kirkham, 1996; Zhang and Schmidt, 1999; Fu and Huang, 2001; Bian and Jiang, 2009; Jiang *et al*., 2010). Although two cultivars of Kentucky bluegrass (*Poa pratensis*) did not differ in POD activities under drought stress, the drought-tolerant cultivar exhibited significantly higher POD activities compared with the sensitive one after rewatering (Xu *et al*., 2011). The results demonstrated complex responses of POD to drought stress, influenced by plant species, cultivar, stress intensity and duration.

In spite of their role in detoxification, PODs have remarkable catalytic versatility involved in a broad range of physiological and developmental processes, including initiation of seed germination (Morohashi, 2002), cellular growth and cell wall loosening (Cosgrove, 2001), cell wall cross-linking (Passardi *et al*., 2004*b*), lignification and suberization (Lopez-Serrano *et al*., 2004), differentiation (Kim *et al*., 2004), senescence (Ranieri *et al*., 2000) and plant–pathogen and plant–insect interactions (Delannoy *et al*., 2003; Gulsen *et al*., 2010*a*). Existing as isoenzymes in plant species, the class III plant POD is a haem-containing glycoprotein encoded by a large number of superfamily genes in land plants (Welinder, 1992*a*, *b*; Hiraga *et al*., 2001). For example, the *Arabidopsis* genome contains 73 genes encoding POD (Tognolli *et al*., 2002), while rice has 138 (Passardi *et al*., 2004*a*). The homology between paralogues in a plant ranges from 30 to 100 %, but very close orthologues exist even between evolutionarily distant plants (Bakalovic *et al*., 2006). More recently, Wang *et al*. (2015) identified 119 non-redundant POD genes in maize, which were divided into 18 groups based on their phylogenetic relationships. Although plant PODs are a family of related proteins possessing highly conserved domains, grasses contain some unique POD clusters not seen in dicot plants, represented by *Arabidopsis* (Duroux and Welinder, 2003). By using *POD* gene polymorphism, genotypic diversity has been examined among *Cynodon* accessions (Gulsen *et al*., 2009), apple (*Malus domestica*) germplasm (Gulsen *et al*., 2010*b*), and Buffalograss (*Bouteloua dactyloides*) and other perennial grass species (Gulsen *et al*., 2007). Peroxidase profiling also reveals genetic linkage between *POD* gene clusters and basal host and non-host resistance to rusts and mildew in barley (*Hordeum vulgare*) (González *et al*., 2010). These results demonstrate that the *POD* gene family can be used to study genotypic diversity and evolutionary relationships on an intra- and inter-specific basis. Senescence- and drought-related changes in two POD isoforms in leaves of *Ramonda serbica* have been observed (Veljovic-Jovanovic *et al*., 2006). Although research results have revealed the functional diversity of PODs, the role of a specific POD isoenzyme in drought tolerance is not clearly determined, especially at the population level for a grass species.

Natural populations are often collected from a wide range of geographical locations that have enormous diversity, which can maximize the potential of populations to withstand and adapt to biotic and abiotic environmental changes (Jump *et al*., 2009). Natural *Brachypodium* accessions broadly group into winter and spring annuals, and this may impact plant responses to environments. Luo *et al*. (2011) found that 57 *Brachypodium* natural accessions varied considerably in whole-plant responses to drought stress and they were classified into most tolerant, moderately tolerant, susceptible and most susceptible groups. Comparative analysis of the cold acclimation and freezing tolerance capacities of seven diploid *Brachypodium* accessions revealed only a limited capacity to develop freezing tolerance when compared with winter varieties of temperate cereals such as wheat (*Triticum aestivum*) and barley (Colton-Gagnon *et al*., 2014). In addition to stress tolerance, Schwartz *et al*. (2010) reported that *VRN* (vernalization regulator) and a portion of the phenotypic variation of flowering time and vernalization was associated with changes in expression of orthologues of *VRN* genes in *Brachypodium* accessions. Variation of phenotypic traits such as plant height, growth habit, stem density, flowering time, seed weight and cell wall composition were also observed among inbred lines (Tyler *et al*., 2014). Natural populations of *Brachypodium* exhibit distant genetic distance patterns (Vogel *et al*., 2009); however, the mechanisms of environmental adaptation during evolution are not well understood in *Brachypodium*. For example, it is not well understood whether some candidate genes encoding POD contribute to variable drought responses of natural accessions.

Although it can be challenging to determine the functions of a large number of isoenzymes in a single plant species, accumulation of information on individual genes should lead to a better understanding of the role of POD in natural variation of stress tolerance and related physiological processes. It is important to know whether allelic variation of genes encoding the specific isoenzymes is associated with plant response to stressful environments. For example, PODs appear to be associated with plant disease resistance based on the presence or absence of isozymes in resistant vs. susceptible varieties. Despite these associations, there is no evidence that the allelic variation of POD directly determines the level of disease resistance (González *et al*., 2010). Therefore, the objectives of this study were to examine natural variation of the POD isoenzyme, to identify nucleotide diversity of *POD* genes and to relate the allelic variation of genes to traits of diverse *Brachypodium* accessions. Knowledge obtained about *Brachypodium* natural variation of PODs increases our understanding of stress resistance among grass species or ecotypes within a species that have evolved in different geographic and adaptive conditions.

MATERIALS AND METHODS

Plant materials and growing conditions

For examining drought tolerance and isoenzymes, drought-tolerant (T-9) and intolerant (B2C) accessions of *Brachypodium* were propagated with the tillers in plastic pots containing a sandy-loam soil with a pH of 6·9 in a greenhouse at Purdue University, West Lafayette, IN, USA. Each pot had the same volume of soil and number of plants. Seedlings were

grown in a greenhouse with a 12 h photoperiod at average temperatures of 23/20 °C (day/night). The average intensity of photosynthetically active radiation (PAR) was approx. 415 µmol m^{-2} s^{-1} during the experiment. Plants were watered every 2 d and fertilized once a week with a soluble fertilizer (N-P_2O_5-K_2O, 24-8-16) (Scotts Inc., Marysville, OH, USA) and micronutrients at the rate of approx. 0·25 g nitrogen L^{-1}. No nutrient deficiency symptoms were observed for any of the plants. The selection of these two accessions was based on our previous study of whole-plant drought response of *Brachypodium* (Luo *et al*., 2011). Subsequently, 34 accessions were selected for detecting POD isoenzyme pattern, population structure, and gene and trait association (Table 1), based on maximization of the geographic and genetic diversity available in these materials (Vogel *et al*., 2009) as well as phenotypic variation in drought responses (Luo *et al*., 2011). With the exception of B21, B3-1 and B2-3 from Iraq, all accessions were from Turkey, which lies in the centre of the natural range of *Brachypodium* and contains all of the environments *Brachypodium* inhabits, including co astal regions, hot interior deserts and cold northern highlands (Vogel *et al*., 2009). Growth conditions of the 34 *Brachypodium* accessions were the same as the conditions for growing T-9 and B2C described above.

Drought treatment

All pots of T-9 and B2C were kept well watered until drought stress was initiated. Drought stresses started on 6 April 2010 after grasses were grown for 21 d from tiller propagation on 16 March 2010. Drought stress was imposed by withholding water from the grasses until permanent wilting (the leaves were no longer rehydrated at night and in the morning) occurred to the plants, especially for the intolerant plants. Drought stress ended on 12 April 2010, at 7 d after stress was initiated. Leaves and roots were harvested at the end of drought treatment and kept frozen (–80 °C) for further analysis.

The experiment was a randomized complete block design with four replicates. The well-watered and drought-stressed grass pots were arranged randomly within a block. Data were analysed for the significance of the treatments for a given measurement using Statistical Analysis System (version 9.1; SAS Institute, Cary, NC, USA). The means of the treatments were separated using the Least Significant Difference (LSD) at a 0·05 significant level.

Table 1. *Experiment identification (ID) and drought tolerance (DT) of* Brachypodium distachyon *accessions used in this study*

ID	Abbreviation	Accession*	DT†	ID	Abbreviation	Accession	DT
34	T-9	Tek-9	T	19	B30-1	Bd30-1	S
31	T-10	Tek-10	T	17	B2K	BdTR2K	S
4	B1-1	Bd1-1	T	16	B2D	BdTR2D	S
27	G-5	Gaz-5	M	26	B9K	BdTR9K	S
8	B13A	BdTR13A	M	22	B7B	BdTR7B	S
9	B13E	BdTR13E	M	5	B10C	BdTR10C	S
1	A-10	Adi-10	M	10	B13M	BdTR13M	S
2	A-14	Adi-14	M	13	B21	Bd21	S
3	A-2	Adi-2	M	28	Kh-1	Kah-1	S
32	T-2	Tek-2	M	29	Kh-3	Kah-3	S
33	T-7	Tek-7	M	30	Koz-4	Koz-4	S
77	B11C	BdTR11C	M	15	B2C	BdTR2C	MS
6	B11A	BdTR11A	M	14	B2B	BdTR2B	MS
24	B8I	BdTRB8I	M	25	B9B	BdTR9B	MS
23	B8C	BdTR8C	M	18	B3-1	Bd3-1	MS
21	B3R	BdTR3R	M	12	B2-3	Bd2-3	MS
20	B3J	BdTR3J	M	11	B18-1	Bd18-1	MS

*B21, B3-1 and B2-3 are from Iraq; all other accessions are from Turkey (Vogel *et al*., 2009).

†T, M, S and MS indicate the most tolerant, moderately tolerant, susceptible and most susceptible accessions, respectively.

Results were based on a study by Luo *et al*. (2011).

Whole-plant measurements

Leaf wilting was visually rated on a scale of 0 (no observable wilting) to 3 (severely wilted or almost dead) (Luo *et al*., 2011). Leaf photochemical efficiency was determined by measuring chlorophyll fluorescence (F_v/F_m) in the dark on randomly selected leaves in each pot using a fluorescence meter (OS-30P, OPTI-Sciences, Hudson, NH, USA). At the end of drought stress (7 d), approx. 3 g of leaf and 5 g of soil were collected, and leaf water content (LWC) and soil water content (SWC) were measured according to the equation: WC = (f. wt – d. wt)/f. wt × 100, where f. wt is fresh weight and d. wt is dry weight for leaf or soil samples, respectively.

Assay of hydrogen peroxide and malondialdehyde

The H_2O_2 content was determined according to the method of Bernt and Bergmeyer (1974) with some modifications. A 0·1 g powder sample of leaf or root was homogenized in 0·4 mL of 100 mM sodium phosphate buffer (pH 6·8), and extracts were then centrifuged at 16 000 *g* for 15 min at 4 °C. A 0·17 mL aliquot of supernatant was added to 0·83 mL of peroxidase reagent containing 83 mM sodium phosphate (pH 7·0), 0·005 % (w/v) *o*-dianisidine and 40 µg peroxidase mL^{-1}. The mixture was incubated at 30 °C for 10 min, and 0·17 mL of 1 N perchloric acid was added to stop the reaction. The absorbance was read at 436 nm. The H_2O_2 concentration was calculated by using a standard curve with known concentration.

Malondialdehyde has been widely used as a convenient biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids because of its facile reaction with thiobarbituric acid (TBA) (Esterbauer and Cheeseman, 1990; Ayala *et al*., 2014). The concentration of MDA was determined to assess the degree of lipid peroxidation using the method of Dhindsa *et al*. (1981) with minor modification. An 80 mg leaf or root sample was homogenized in 1·2 mL of 1 % trichloroacetic acid. The mixture was centrifuged at 10 000 *g* for 10 min. A 0·5 mL aliquot of supernatant was added to 2 mL of reaction solution (20 % trichloroacetic acid containing 0·5 % TBA). The mixture was heated at 95 °C for 30 min, quickly cooled in an ice bath, and then centrifuged at 10 000 *g* for 10 min. The absorbance was read at 532 and 600 nm.

POD activity and isoenzyme detection

For leaf enzyme extraction, 0·1 g of ground powder sample was homogenized with 1·2 mL of extraction buffer [50 mM potassium phosphate, 1 mM EDTA, 1 % polyvinylpyrrolidone

(PVP), pH 7·8). For root enzyme extraction, 0·12 g of powder sample was homogenized with 0·72 mL of the same extraction buffer. The mixtures were centrifuged at 15 000 g for 25 min at 4 °C and the supernatant was collected. Protein content was determined according to Bradford (1976). POD activity was determined by following changes in absorbance at 470 nm (Bian and Jiang, 2009). Details of the methods were described by Zhang and Kirkham (1996).

Electrophoretic separation of isoenzymes was performed using non-denaturing PAGE described by Laemmli (1970), except that SDS was omitted; 8 % stacking and 3 % resolving polyacrylamide gels were used with running buffer at pH 8·5. Equal amounts of protein (30 µg) for the leaf samples were loaded. Native gels were stained for POD activity according to the method of Srivastava and Van Huystee (1977) with some modifications. Briefly, gels were soaked in 100 mM potassium phosphate buffer (pH 6·5) for 15 min and then stained in 12·5 mM guaiacol containing 12 mM H_2O_2.

Protein identification by mass spectrometry

Two mass spectroscopy techniques, matrix-assisted laser desorption (MALDI) (Baumgarner *et al*., 2013) and liquid chromatography-electrospray ionization (ESI) tandem mass spectrometry (LC-ESI-MS/MS) (Park *et al*., 2012), were used for protein identification. Briefly, distinct isoenzymes of POD from the selected drought-tolerant and susceptible accessions were recovered from a native PAGE gel. The bands were excised from the gel and processed in the Purdue University Proteomics core facility in the Bindley Biosciences Center for in-gel digestion (Jiménez *et al*., 1998) and then analysed by MADLI and LC-ESI-MS/MS analysis. The digested peptide solution was spotted on the plate, and peptide identification was performed using MALDI time-of-flight/time-of-flight (TOF/TOF) on an AB 4800 Plus Analyzer (Applied Biosystems) (Baumgarner *et al*., 2013). MALDI data were analysed using GPS Explorer™ Software (Applied Biosystems). Searches were performed using MS and MS/MS data against databases for *Brachypodium*, rice, *Arabidopsis* and green plants from the National Center for Biotechnology Information (NCBI). In addition, the digested peptides were extracted and separated in a nano-HPLC system (Agilent 110, Agilent Technologies, San Jose, CA, USA), coupled with an ESI hybrid linear ion trap quadrupole Orbitrap mass spectrometer (ESI-LTQ-Orbitrap XL) (Park *et al*., 2012). Peptides were separated with a C18 reversed phase ZORBAX 300SB-C18 analytical column (150 mm × 75 µm, 3·5 µm) from Agilent. The peptides were eluted from the column with a linear gradient of 5–40 % buffer B (acetonitrile/0·1 % formic acid) in buffer A (water/0·1 % formic acid) over 35 min at a rate of 0·3 µL min^{-1} followed by a gradient of 40–95 % B for 40–55 min. The column was equilibrated with 5 % buffer B (acetonitrile/0·1 % formic acid) for 55–70 min. The MS spectra were acquired in positive mode in a range from 300 to 2000. The MS/MS spectra were acquired in a data-dependent acquisition mode in which the full MS scan (resolution 30 000) was followed by four MS/MS scans for the most abundant molecular ions and fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35 %. Peptide data (MS/MS) information from LTQ-Orbitap were analysed using the AB 4800 Plus/Protein Pilot software (Applied Biosystems) against the SwissProt database. Peptides with similarity values >95 % were considered toward identification of a given protein, with a minimum of two peptides required for positive identification.

DNA extraction and sequencing

Primers were designed to amplify genomic fragments for each gene (Table 3). Genomic DNA of the 34 accessions was extracted using a modified hexadecyltrimethylammonium bromide protocol (Doyle and Doyle, 1990) and purified using a PureLink™ Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA). PCR amplification of the gene fragment was conducted with one cycle at 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 53 °C for 30 s, 72 °C for 60 s, and a final extension step at 72 °C for 5 min. Bands of the expected size were cut from the gel and recovered using a ZR-96 Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, CA, USA). The recovered DNA was used for sequencing PCRs with a BigDye Terminator kit with one cycle at 96 °C for 1 min, followed by 35 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. After the clean up with ethanol/sodium acetate solution, the purified DNA was sequenced on an ABI 3730XL sequencer (Applied Biosystems Inc., Foster City, CA, USA) at the Genomics Core Facility at Purdue University.

Nucleotide diversity (π) and linkage disequilibrium (LD)

DNA sequence data from 29–34 accessions were aligned by DNASTAR (DNASTAR, Inc., Madison, WI, USA), depending on the quality of sequencing results for the individual genes (Table 4). π, LD and neutrality tests were estimated using DnaSP5.0 (Rozas and Rozas, 1999). The nucleotide level was assayed by nucleotide polymorphism (θ) (Watterson, 1975) and π (Nei, 1987). Values from Tajima's D test were separated by the standard deviation of π and θ_w and reflected the differences between the number of singletons and total number of mutations (Tajima, 1989). Decay of LD with distance in base pairs between sites within the same gene was evaluated by non-linear regression. The LD descriptive statistic r^2 (Hill and Robertson, 1968) was calculated using TASSEL software (Bradbury *et al*., 2007). Neighbor–Joining trees were created based on haplotype sequences for each candidate gene using nucleotide number of differences as a distance measure and were calculated with TASSEL software.

TABLE 2. *Effects of 7 d drought stress on soil moisture content (SWC), leaf water content (LWC) and chlorophyll fluorescence (F_v/F_m) of* Brachypodium distachyon

Accession	Treatment	SWC (%)	LWC (%)	F_v/F_m
T-9	Control	19·5 ± 0·62a*	77·3 ± 0·45a	0·81 ± 0·002a
	Drought	5·5 ± 0·34b	59·3 ± 4·46b	0·81 ± 0·003a
B2C	Control	18·7 ± 1·01a	76·0 ± 0·33a	0·82 ± 0·005a
	Drought	4·4 ± 0·19b	46·2 ± 8·92b	0·78 ± 0·017b

Values are mean ± s.d.

*Means followed by the same letter within a column for a given line were not significantly different at $P < 0·05$.

Association analysis of single nucleotide polymorphisms (SNPs) with traits

The population structure was assessed using 46 simple sequence repeats (SSRs) (Vogel *et al*., 2009) by STRUCTURE software (Pritchard *et al*., 2000); the detailed procedure was described previously (Yu *et al*., 2013). Individual SNPs with each trait were tested with the simple linear model and population structure implemented model using TASSEL software (Bradbury *et al*., 2007). SNP markers with minor allele frequency <5 % were filtered from the association analysis. The SNPs that passed $P < 0{\cdot}05$ were deemed significant.

Gene expression

Real-time quantitative reverse transcription–PCR (qRT–PCR) was conducted to examine gene expression in the control and drought-stressed leaf samples of T-9 and B2C. Briefly, total RNA was isolated with a Direct-zol™ RNA MiniPrep Kit (Zymo Research Corporation) and then used for reverse transcription with an iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Reaction mixtures were incubated for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. qRT–PCR was performed with a CFX96 Touch™ Real-Time PCR Detection System using an iTaq™ Universal SYBR® Green Kit (Bio-Rad), with reaction for 3 min at 95 °C followed by 40 amplification cycles of 10 s at 95 °C and 1 min at 60 °C. Primers for amplification were as follows: for *Bradi3g41340.1*, Forward 5′-GCAGCAACCTG GAGAAGAT-3′ and reverse 5′-GGCAGTCGTGGAAGAA GAG-3′; for *Bradi1g26870.1*, Forward 5′-TCTTCGGTTCT CTCCTTCCT-3′ and reverse 5′-ATCGCAGGCTTCCT TGTT-3′. The transcript level of *S*-adenosylmethionine decarboxylase (*SamDC*) was used as a housekeeping gene (Hong *et al*., 2008). The method of $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) was used to calculate the expression level under drought-stressed conditions relative to that under control conditions. The analysis included three biological replicates (three pots) and three technical replicates for each accession under control and drought stress conditions.

RESULTS

Whole-plant response to drought stress

The SWC dropped from 19·5 to 5·5 % for T-9 and from 18·7 to 4·4 % for B2C under drought stress (Table 2). The reductions in

TABLE 3. *Primers for gene amplification in* Brachypodium distachyon

Gene	Forward primer	Reverse primer
Bradi1g41900.1	5′-CATCCGCATCTTCTTCCACGA-3′	5′-TTGACCATCCGGCAATTCCT-3′
Bradi2g04490.1	5′-ACTGTCCTGATGCCGAGGATA-3′	5′-TTGAAGAAGCTCGCGTCGAA-3′
Bradi3g41340.1	5′-AGGAGATCAAGGCCAAGCTCAA-3′	5′-TACACGATCGAGAACCGATGGA-3′
	5′-TGTTTCCTTCCCTTTTTTCTC-3′	5′-GCTTGGACAGACAGTCGATTA-3′
	5′-ACGAGATTAGTTAAAGCCAAGG-3′	5′-GTTCACGCTCCCCTCATCC-3′
Bradi1g63060.1	5′-AGCTGAGCTGGAACCATCAA-3′	5′-CAGTCGCAAATGCTCCACAA-3′
	5′-TGTCCCTCAACTATTGGCATC-3′	5′-TTCTCAAAACCCTGGTGCTG-3′
	5′-AAGTAGAACATGCCTCCTCGAT-3′	5′-GGTAGGGTGAGGTCCACAAAAA-3′
Bradi1g26870.1	5′-TCGCGAATCCATTTCTCCGA-3′	5′-AAACACCTCCCGTGCATTGT-3′
	5′-GTGCCCTACTGGTCGCTCAA-3′	5′-GGAGCGAAGTAGCACGCAGTA-3′
Bradi4g32800.1	5′-TCCAGTTCCATGACTGCTTCGT-3′	5′-AAGGCATTCTGGTCCTTGCT-3′
Bradi5g00690.1	5′-AAATCGCCATTGGCCCGTAT-3′	5′-TACTGGTTGTCGAACCGGAACT-3′
Bradi1g65820.2	5′-TTTACCTCCCAGTAGCAGCCAT-3′	5′-TTCCAAGAGCAACCAACCCA-3′

TABLE 4. *Gene length, sequence length, single nucleotide polymorphism (SNP) and SNP frequency in the whole length and coding region of peroxidase genes*

Gene	*n*	Whole length (bp)	CDS for gene (bp)	Sequencing length (bp)	CDS for sequencing (bp)	Sequencing SNP number	Coding SNP number	Whole SNP frequency	Coding SNP frequency
Bradi1g41900.1	34	1332	1122	804	737	8	5	101	147
Bradi2g04490.1	29	1608	981	1060	557	14	12	76	46
Bradi3g41340.1	33	2911	1008	1184	151	3	0	395	–
Bradi1g63060.1	32	3176	966	1782	424	12	5	149	85
Bradi1g26870.1	34	1449	1047	1271	1032	9	7	141	147
Bradi4g32800.1	30	1483	1044	841	624	18	10	47	62
Bradi5g00690.1	33	1365	693	670	373	8	5	84	75
Bradi1g65820.2	34	3166	753	1566	395	18	0	87	–
Mean	32	2061	952	1147	537	11	6	135	94

n, the number of accessions for which a certain gene could be sequenced; whole length, the genome sequence of the gene; CDS for gene, the coding region of a gene; sequencing length, the entire sequenced length for a gene in this study; CDS for sequencing, the sequenced length for the coding region in this study; sequencing SNP number, number of SNPd discovered in the entire sequenced length; coding SNP number, number of SNP discovered in the sequenced length for the coding region; whole SNP frequency, calculated by the entire sequenced length/SNP, stands for the average SNP per bp length; coding SNP frequency, the sequenced length for the coding region/SNP, stands for the average SNP per bp length.

SWC under drought stress was >70 % for both accessions, compared with their respective controls. The LWC was significantly reduced from 77·3 to 59·3 % for T-9 and from 76·0 to 46·2 % for B2C after 7 d of drought stress. Drought stress did not change chlorophyll fluorescence (F_v/F_m) for T-9, but significantly decreased F_v/F_m for B2C, compared with the control (Table 2).

The production of H_2O_2 and MDA

Leaf H_2O_2 concentrations were increased by 1·8-fold for T-9 and 2·5-fold for B2C but remained unchanged in the roots for both accessions under drought stress (Fig. 1). The MDA concentrations were increased by 2·8- and 3·5-fold in the leaves for T-9 and B2C, respectively, compared with their controls; but remained unchanged in the roots for both accessions under drought stress (Fig. 1).

Enzyme activity and isoenzyme stain

Compared with the control, drought stress increased leaf POD activities by 2·0-fold for T-9 and 1·6-fold for B2C, but did not affect root POD activities in both accessions (Fig. 1). Based on the non-denaturing PAGE protein gel, drought stress did not induce new anionic POD isoenzymes (pH 8·5) in either the tolerant accession T-9 or the susceptible B2C (Fig. 2). Both T-9 and B2C had their one own unique isoenzyme not shown in the other. Across the other 32 accessions, the drought-tolerant accessions T-2, T-7, T-10, Bd1-1, B8C and B8I had the same POD isoenzyme patterns as T-9, while the drought-susceptible accessions such as BD18-1, B2B, B9B and B7B had similar isoenzyme patterns to B2C (Fig. 3). Two cationic POD isoenzymes (pH 4·5) were also detected, but the patterns were the same among accessions (data not shown).

Protein and SNP identification

Protein sequences revealed that isoenzyme 1 in T-9 contained four different proteins (Bradi1g41900.1, Bradi2g04490.1, Bradi3g41340.1 and Bradi1g63060.1), while isoenzyme 2 in B2C also contained four different proteins (Bradi1g26870.1, Bradi4g32800.1, Bradi5g00690.1 and Bradi5g65820.2)

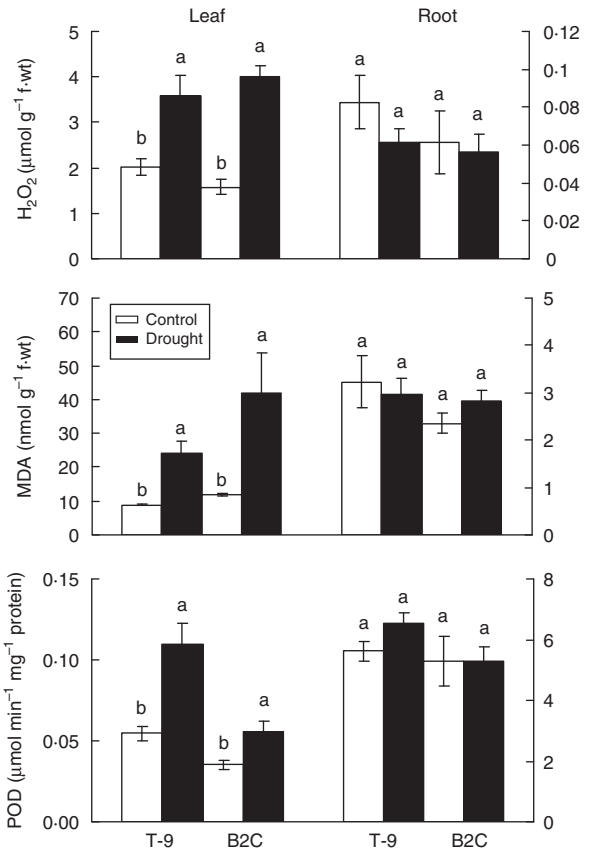

Fig. 1. Effects of 7 d drought stress on the concentration of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) and activities of peroxidase (POD) in the leaves and roots of *Brachypodium distachyon*. Means followed by the same letter within the leaves or roots for a given line are not significantly different at $P < 0·05$. Vertical bars show ± s.d. T-9, Tek-9; B2C, BdTR2C.

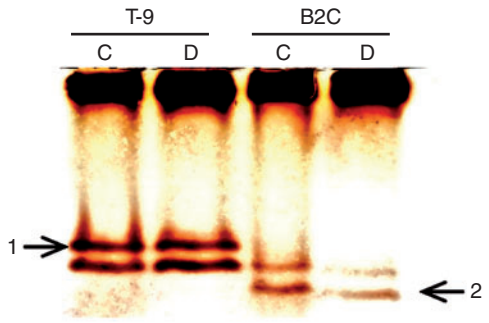

Fig. 2. Electrozymogram showing the peroxidase profile of protein extracts from leaves of *Brachypodium distachyon* under well-watered control (C) and drought stress (D) conditions. Arrows indicate unique bands shown in the drought-tolerant T-9 (isoenzyme 1) and susceptible B2C (isoenzyme 2) accessions. T-9, Tek-9; B2C, BdTR2C.

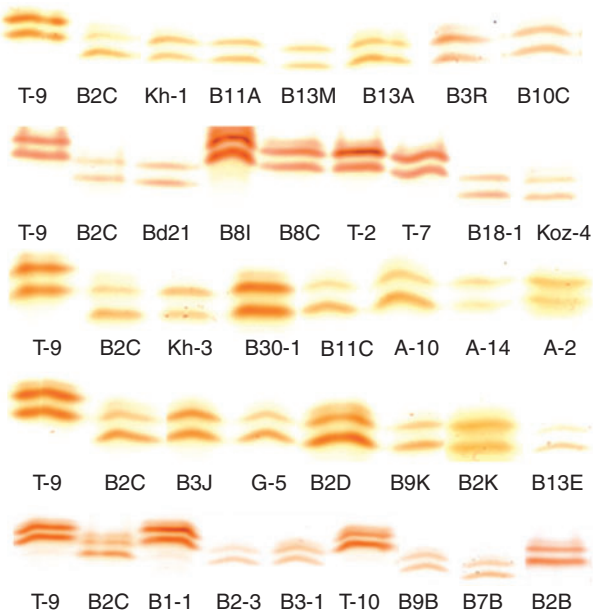

Fig. 3. The total activity stain for peroxidase in the leaves of *Brachypodium distachyon* under well-watered conditions for accessions used in the study.

(Supplementary Data Tables S1 and S2). Genes of *Bradi1g41900.1*, *Bradi2g04490.1*, *Bradi5g41340.1*, *Bradi1g26870.1*, *Bradi4g32800.1* and *Bradig00690.1* were identified through MALDI-TOF/TOF, while genes of *Bradi63060.1* and *Bradi65820.2* were identified by LTQ-Orbitrap (Tables S1 and S2). The genes encoding these proteins were identified and partially sequenced in genotypes with contrasting drought tolerance (Table 4). The total length of the sequences ranged from 670 to 1782 bp among eight genes, with a mean of 1147 bp, and covered approx. 41–88 % of the whole length of the genes (Table 4). The sequences of coding regions ranged from 151 to 1032 bp among the eight genes, with a mean of 546 bp. A total of 90 SNPs were detected in the eight *POD* genes, ranging from three (*Bradi3g41340.1*) to 18 SNPs (*Bradi4g32800.1* and *Bradi5g65820.2*). The coding regions consisted of 44 SNPs ranging from 0 (*Bradi3g41340.1* and *Bradi5g65820.2*) to 12 (*Bradi2g04490.1*). Across all the genes, the average SNP frequency was 1/135 bp, ranging from one SNP per 46 bp (*Bradi4g32800.1*) to one SNP per 395 bp (*Bradi3g41340.1*). The coding regions had an average SNP frequency of 1/94 bp (Table 4).

π and LD

Across eight genes, the values of π ranged from 0·00 (*Bradi3g41340.1* and *Bradi5g65820.2*) to 0·0077 (*Bradi4g32800.1*) and Watterson's θ_w ranged from 0·00 (*Bradi3g41340.1* and *Bradi5g65820.2*) to 0·0061 (*Bradi4g04490.1*), with a mean value of 0·0028 for π and 0·0019 for θ_w, respectively (Table 5). The π values spanned from 0·00 (*Bradi3g41340.1*) to 0·0055 (*Bradi5g00690.1*) of synonymous areas with an average of 0·0019, and ranged from 0·0011 (*Bradi1g26870.1*) to 0·0095 (*Bradi3g041340.1*) of non-synonymous areas with an average of 0·0042 (Table 5). The average π synonymous/non-synonymous ratio was 0·81 (Table 5). The haplotype number varied from four (*Bradi3g41340.1*) to 16, with an average of 9·1 across genes. Haplotype diversity ranged from 0·51 (*Bradi1g41900.1*) to 0·91 (*Bradi4g32800.1*), with a mean value of 0·65. Through the neutrality test, positive Tajima's D values were shown in *Bradi3g41340.1*, *Bradi4g32800.1*, *Bradi5g00690.1* and *Bradi5g65820.2*, while negative Tajima's D values were found in *Bradi1g41900.1*, *Bradi2g04490.1*, *Bradi3g63060.1* and *Bradi1g26870.1*.

Population structure and LD

Two genetic population structures (G1 and G2) were found in 33 genotypes assessed by 46 SSR markers (B30-1 was not included due to missing SSR information) (Fig. 4). The subgroups were identified based on likelihood plots of the models, stability of grouping patterns across different runs, and germplasm information (Vogel *et al*., 2009; Yu *et al*., 2013). G1 consisted of T-9, T-10, B1-1, T-2, T-7, B8I, B8C and B7B accessions with better drought tolerance, except for B7B, while G2 contained other accessions varying in drought tolerance but including the most susceptible materials. Based on our previous trait data in these accessions (Luo *et al*., 2011), G1 had an average value of 0·78, 56·3 and 30·6 % for leaf wilting, percentage

TABLE 5. *Nucleotide diversity, haplotype and neutrality test of peroxidase genes in* Brachypodium distachyon

Gene	π	θ_w	π_{syn}	$\pi_{non\text{-}syn}$	$\pi_{syn/non\ syn}$	H	Hd ± s.d.	Neutral test	Tajima's D
Bradi1g41900.1	0·0003	0·0011	0·0009	0·0025	0·366	6	0·51 ± 0·089	–0·529	–0·603
Bradi2g04490.1	0·0031	0·0061	0·0011	0·0022	0·498	15	0·82 ± 0·074	–1·847	–1·564
Bradi3g41340.1	0·0000	0·0000	0·0000	0·0095	0·000	4	0·49 ± 0·091	1·060	0·856
Bradi1g63060.1	0·0026	NA	0·0032	0·0014	2·331	9	0·61 ± 0·097	0·041	–2·139
Bradi1g26870.1	0·0015	0·0025	0·0012	0·0011	1·055	7	0·52 ± 0·093	–2·197	–1·057
Bradi4g32800.1	0·0077	NA	0·0030	0·0086	0·352	16	0·91 ± 0·037	1·363	0·250
Bradi5g00690.1	0·0073	NA	0·0055	0·0029	1·884	7	0·68 ± 0·059	0·380	1·009
Bradi1g65820.2	0·0000	0·0000	0·0002	0·0053	0·0398	9	0·68 ± 0·075	1·066	1·522
Mean	0·0028	0·0019	0·0019	0·0042	0·812	9·1	0·65 ± 0·077	–0·083	–2·156

π and θ_w, nucleotide diversity; π_{syn} and $\pi_{non\text{-}syn}$, synonymous and non-synonymous nucleotide diversity, respectively; H, number of haplotypes; Hd, haplotype diversity; NA, not available.

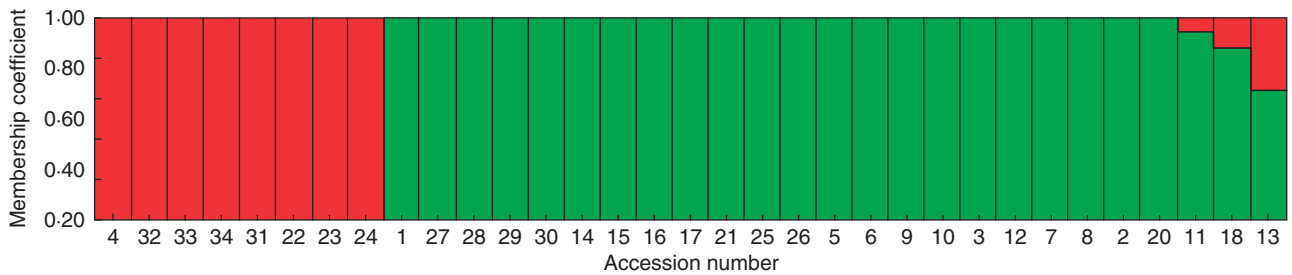

FIG. 4. Hierarchical organization of genetic relatedness of 33 *Brachypodium* genotypes. Numbers on the *x*-axis represent each individual accession. Numbers on the *y*-axis indicate the membership coefficient for each individual accession. The colour of the bar indicates the two groups identified through the STRUCTURE program (G1, red; G2, green).

reduction of LWC (R-LWC) and F_v/F_m (R-F_v/F_m), which was lower than 1·3, 76·7 and 56·7 % for leaf wilting, R-LWC and R-F_v/F_m observed in G2, respectively.

Linkage disequilibrium estimates r^2 plotted against pairwise distances between SNPs. Across genes, slow LD decay was found in *Brachypodium* (Fig. 5). Overall, LD decay extended to > 1·2 kb with an r^2 higher than 0·6. Similarly, LD decay of combined *Bradi1g41900.1*, *Bradi2g04490.1*, *Bradi3g41340.1* and *Bradi3g63060.1* extended to 1·2 kb with an r^2 of 0·6, while *Bradi1g26870.1*, *Bradi4g32800.1*, *Bradi5g00690.1* and *Bradi5g65820.2* expressed LD decay exceeding the distance of 1·2 kb with an r^2 higher than 0·6 (data not shown).

Association of genes with traits

After controlling population structure, significant associations of *Bradi3g41340.1* and *Bradi1g26870.1* with traits were identified (Table 6). Specifically, SNPs at loci 1087 and 1584 from *Bradi3g41340.1* were associated with R-LWC, with nucleotide changes of A/T and GT. Similarly, SNPs at loci 617, 708, 806, 1164 and 1181 from *Bradi1g26870.1* were associated with R-LWC, with nucleotide changes of A/T, C/T, A/G, C/T and G/T. One SNP at locus 1164 from *Bradi1g26870.1* was also associated with leaf wilting. Compared with using the simple linear model, this was an elimination of approx. 87 % significant associations by using the population structure implemented model (data not shown).

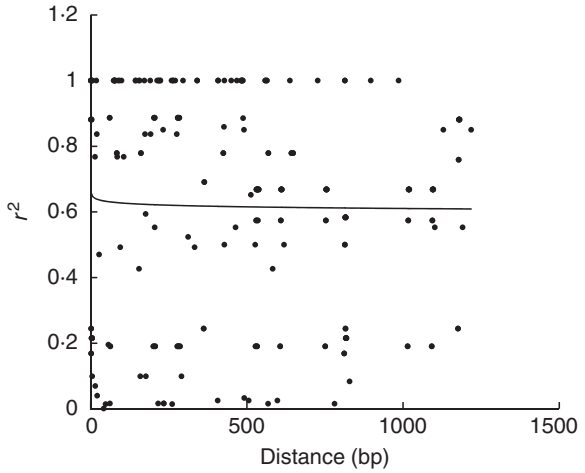

FIG. 5. Pattern of linkage disequilibrium (LD) in eight *POD* genes in *Brachypodium distachyon*. The r^2 values for pairwise LD are plotted against physical distance.

TABLE 6. *Association of peroxidase genes with traits and nucleotide changes in* Brachypodium distachyon

Gene	Trait	Locus	Nucleotide change
Bradi3g41340.1	R-LWC	1087	A/G
	R-LWC	1574	G/T
Bradi1g26870.1	R-LWC	617	A/G
	R-LWC	708	C/T
	R-LWC	806	A/G
	R-LWC	1164	C/T
	R-LWC	1181	G/T
	Wilting	1164	C/T

Wilting, leaf wilting under drought; R-LWC, percentage reduction of leaf water content under drought compared with the control.

Leaf wilting and LWC data were based on a study by Luo *et al*. (2011).

Gene expression

The expression of two significant genes of *Bradi3g41340.1* and *Bradi1g26870*.1 was analysed in the leaves of both accessions under the non-stress and drought conditions (Fig. 6). Relative to their unstressed control, drought stress significantly increased expression levels of *Bradi3g41340.1* and *Bradi1g26870.1* in both accessions. Specifically, expression of *Bradi3g41340.1* was significantly increased by 4·8-fold for T-9 and 3·1-fold for B2C. For *Bradi1g26870.1*, expression was increased by 5·6-fold for T-9 and 4·7-fold for B2C (Fig. 6) However, differences in fold change were not significant between T-9 and B2C. In addition, by using a reference gene as a control, the absolute expression levels of both genes were not significantly different between the two accessions under the unstressed control and drought stress (data not shown).

Phylogenetic tree

The Neighbor–Joining tree revealed certain relationships among the accessions based on sequences of eight genes. Drought-tolerant accessions such as T-2, T-9, T-7, T-10, B1-1, B8I and B8C were close to each other according to sequences

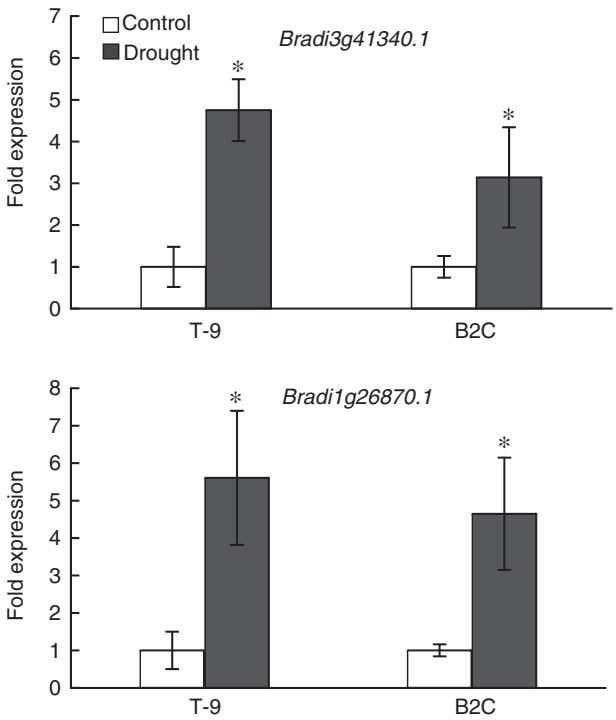

FIG. 6. Effects of 7 d of drought stress on gene expression by using real-time quantitative reverse transcription–PCR in the control and drought-stressed leaf samples of T-9 and B2C. T-9, Tek-9; B2C, BdTR2C. Data were normalized in comparison with the control. *, significant at $P < 0·05$.

of *Bradi3g41340.1*. The results were generally consistent with grouping revealed by population structure using SSR markers (Fig. 7). By using sequences of *Bradi1g26870.1*, grouping was somewhat different from the results from *Bradi3g41340.1*.

DISCUSSION

Lesser reductions in LWC and F_v/F_m found in T-9 compared with B2C exposed to 7 d of drought stress indicated that T-9 was more drought tolerant than B2C. The results were consistent with a study in *Brachypodium* by Luo *et al.* (2011) who supported that more reductions in LWC and F_v/F_m were observed in B2C. Drought treatment was less severe in this experiment compared with that described by Luo *et al.* (2011). This could be the main reason for the relatively small decreases in LWC, and particularly F_v/F_m, in the present study when compared with previous results. However, the trends in drought responses of the two accessions were similar between the two studies. A more drought-tolerant perennial ryegrass (*Lolium perenne*) also showed higher LWC and F_v/F_m in the field (Yu *et al.*, 2013), suggesting that drought-tolerant grass plants maintain adequate water status and physiological activity.

Moreover, the drought-tolerant T-9 also showed more increased POD activities and lower H_2O_2 and MDA concentrations, relative to the control. Similar results of increased POD activity were found in the drought-tolerant maize (Moussa and Abdel-Aziz, 2008). However, POD activity also remained unchanged in some grass species under drought stress (Zhang and Kirkham, 1996; Bian and Jiang, 2009; Jiang *et al.*, 2010). Changes in POD activity under drought stress may depend on the species, variety, duration and intensity of the stress. At the transcript level, expression of the two significant genes, *Bradi3g41340.1* and *Bradi1g26870.1*, was increased in T-9 and B2C under drought stress, relative to their control (Fig. 6). The results were consistent with the increased POD activities found in these two accessions under drought stress (Fig. 1). The increases in enzyme activity and gene expression indicated that enhanced POD plays a role in drought tolerance of *Brachypodium*, which could be associated with reducing oxidative injury. In addition, some POD genes were up-regulated and some were down-regulated in maize due to drought stress (Wang *et al.*, 2015). Higher activities of POD and gene expression were also found in plants exposed to other abiotic stresses such as cold, salt stress, heavy metals and pathogen invasion (Hiraga *et al.*, 2000; Sharma and Dubey, 2004; Azevedo Neto *et al.*, 2006; Xu *et al.*, 2008).

Drought stress could also affect POD isozymes in some plants species. Dehydration and senescence caused disturbance in the redox homeostasis of *Ramonda* leaves, while inducing different POD anionic and cationic isoenzymes (Veljovic-Jovanovic *et al.*, 2006). In *Arabidopsis*, the intensity of two POD isoenzymes increased in drought-stressed young and mature leaves, especially a strong induction in one isoenzyme in mature leaves (Jung 2004). Some PODs were stress-responsive isoenzymes in the roots of wheat (*Triticum aestivum* L.) (Selote and Khanna-Chopra, 2010). In this study, T-9 and B2C showed their own unique isoenzyme of POD under normal conditions.

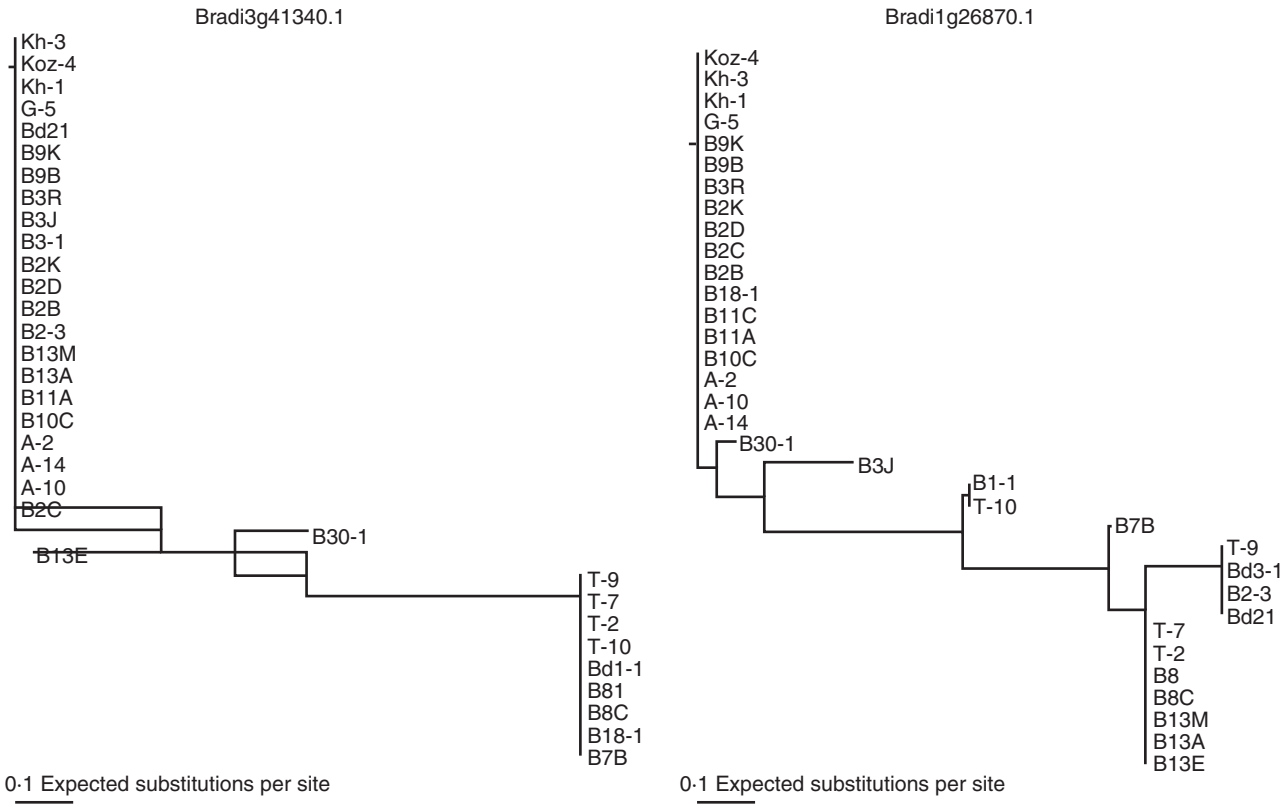

FIG. 7. Neighbor–Joining trees representing gene haplotype relationships in *Brachypodium distachyon*.

Although drought stress did not induce new bands of POD, these specific POD isoenzymes can generally distinguish the more drought-tolerant accessions from the others (Table 1; Fig. 3). The results indicated that the POD isoenzyme could differentiate *Brachypodium* accessions with contrasting drought tolerance. Among those POD isoenzymes, two candidate genes of *Bradi3g41340.1* and *Bradi1g26870.1* encoding specific PODs were significantly associated with drought tolerance traits. Furthermore, the grouping of the drought-tolerant accessions by sequences of *Bradi3g41340.1* was generally consistent with grouping revealed by population structure using genome-wide SSR markers. The results illustrated relationships among certain accessions by POD sequences, which could separate the most drought-tolerant accessions from the other accessions.

The average SNP frequency in eight *POD* genes observed in this study was one SNP every 135 bp in whole sequencing length. It was very similar to that of other antioxidant genes in *Brachypodium* (Luo *et al*., 2012). However, the average SNP frequency (1 SNP per 180 bp) in *Bradi1g41900.1*, *Bradi2g04490.1*, *Bradi3g41340.1* and *Bradi3g63060.1* initially found in the tolerant accession was only half compared with *Bradi1g26870.1*, *Bradi4g32800.1*, *Bradi5g00690.1* and *Bradi5g65820.2* shown in the susceptible accession. Moreover, the significantly associated genes of *Bradi3g41340.1* and *Bradi1g26870.1* had even lower SNP frequency, suggesting that SNP frequency in *POD* genes varied widely in one species. It indicated that SNP frequency may be influenced by selection, mutation, mating system, effective population size and demography (Nei, 1987). Mean nucleotide diversity ($\pi = 0{\cdot}0028$, $\theta_w = 0{\cdot}0019$) of *POD* genes was also similar to the values found in other antioxidant genes in *Brachypodium* (Luo *et al*., 2012) and in balsam poplar (*Populus balsamifera*) (Olson *et al*., 2010). The range of π values in *Brachypodium* was similar to the range of those of other genes tested in 34 ecotypes of arabidopsis (Lin *et al*., 2008). The value of θ_w denotes the number of polymorphic segregating sites (Watterson, 1975). The difference between π and θ_w found in *Bradi1g41900.1*, *Bradi2g04490.1* and *Bradi1g26870.1* could reflect the degree of non-equilibrium conditions in the genetic history of the population. Tajima's D measures the standardized differences between π and θ_w; negative D values might indicate an excess of low-frequency polymorphisms in *Bradi1g41900.1*, *Bradi2g04490.1*, *Bradi3g63060.1* and *Bradi1g26870.1*, whereas positive values indicated an excess of intermediate polymorphisms in *Bradi3g41340.1*, *Bradi4g32800.1*, *Bradi5g00690.1* and *Bradi5g65820.2*. The ratio between $\pi_{non\text{-}syn}$ and π_{syn} is a strong indicator of selection (Li, 1997), varying greatly in these *POD* genes in *Brachypodoum*. A higher $\pi_{non\text{-}syn}$ value in *Bradi3g41340.1* suggested a lack of selection of pressure during evolution in maintaining a high level of polymorphisms.

In summary, POD activity increased in the leaf of *Brachypodium* under drought stress, to a greater extent in the tolerant accession T-9, along with lesser accumulation of MDA and H_2O_2. Variations of POD isoenzymes were found among accessions with contrasting drought tolerance, while the most tolerant and susceptible accessions had their unique POD isoenzyme band. Eight *POD* genes were identified and a total of 90 SNPs were found across 34 accessions. Nucleotide diversity of *POD* genes ranged from 0·00 to 0·0077 with a mean of 0·0028, and the average synonymous/non-synonymous π ratio was 0·81. After controlling population structure, significant associations of *Bradi3g41340.1* and *Bradi1g26870.1* with R-LWC or leaf wilting were identified. The results suggested a role for specific *POD* genes in differentiating *Brachypodium* accessions with contrasting drought tolerance, which could be associated with general fitness of *Brachypodium* during evolution.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: the proteins identified by using MALDI-TOF/TOF MS in Tek-9 and BdTR2C *Brachypodium distachyon* accessions. Table S2: the proteins identified by LC-ESI-LTQ-Orbitrap XL in Tek-9 and BdTR2C *Brachypodium distachyon* accessions

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