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Research article

The barley transcription factor HvMYB1 is a positive regulator of drought tolerance

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ABSTRACT

Transcription factors such as MYB have previously been associated with the plant response to drought. In this work, studies on the function of the barley (*Hordeum vulgare* L.) transcription factor *HvMYB1* show that gene expression is upregulated in wildtype barley roots and leaves under drought and osmotic stress. Transgenic barley plants that overexpress *HvMYB1* were found to be more resistant to drought, showing enhanced relative water content and reduced water loss rate and stomatal conductance as compared to control plants. Levels of the osmolyte proline were enhanced as was expression of dehydrin *HvDNH6* in the transgenic lines under drought conditions. The levels of the reactive oxygen species H_2O_2 were enhanced in wildtype roots and leaves by drought, but less so in the *HvMYB1* overexpressing lines. Enzyme activity of the low affinity H_2O_2 degrading enzyme catalase (EC 1.11.1.6) was also lower in droughted *HvMYB1* overexpressing lines. Gene expression of the high affinity ROS scavengers *ASCORBATE PEROXIDASE* and *GLUTATHIONE PEROXIDASE* was found to be constitutively high in the overexpressing lines, whereas *CATALASE* gene expression was similar to the control plants. These results suggest a role for HvMYB1 in protecting plants against drought in the vegetative plant by acting as a mediator of abscisic acid action.

1. Introduction

Drought stress is caused by limited water supply to roots, and is compounded by elevated transpiration rates due to high temperatures. Drought results in loss of turgor and reduction in photosynthetic capacity causing impaired plant growth, reduced yield and quality of the harvested product. Another important aspect of drought is the enhanced production in the plant of reactive oxygen species, leading to oxidative stress (de Carvalho, 2008; Gill and Tuteja, 2010; Noctor et al., 2014). Plants have various adaptive mechanism to cope with drought stress which act at the molecular, cellular, morphological and whole plant physiology level, and numerous studies have set out to elucidate how these complex pathways and control mechanisms operate (e.g. Golldack et al., 2014; Todaka et al., 2015). In particular, large scale gene expression studies have identified a number of the genes regulated in response to drought (Seki et al., 2001; Zhang et al., 2017; Cantalapiedra et al., 2017). Among those genes are a number that encode transcription factors. These transcription factors have been found to belong to many different families including bZIP, NAC, WRKY, MYC, zinc finger and MYB (Yamaguchi-Shinozaki and Shinozaki, 2006; Abe et al., 2003).

MYB proteins are eukaryotic transcription factors first identified in avian myeloblastoma virus (Klempnauer et al., 1982) and subsequently in vertebrates (Weston, 1998) and in plants, such as barley (Marocco et al., 1989). MYB proteins are characterised by conserved N-terminal MYB DNA binding domains; different classes of MYB protein have different numbers of MYB domains, and in plants the majority of the MYB proteins have two such domains and are referred to as R2R3 MYB proteins. The R2R3 MYBs are encoded by multigene families; Arabidopsis has up to 190 R2R3 encoding genes (Yanhui et al., 2006), maize has 157 MYB R2R3 genes (Du et al., 2012) and barley has at least 51 R2R3 MYB genes (Tombuloglu et al., 2013). The plant R2R3 Myb proteins have been associated with a number of different processes in plants, including primary and secondary metabolism, cell differentiation and development but also responses to biotic and abiotic stress (reviewed Ambawat et al., 2013; Roy, 2016). A number of studies have shown that MYB gene expression in cereals is associated with abiotic stress. Determination of function for MYB proteins has largely been

Abbreviations: ABA, abscisic acid; PEG, polyethylene glycol; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction

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inferred through analysis of *MYB* gene expression patterns or by overexpression expression in the model plant species Arabidopsis and tobacco. Gene expression studies indicate that many wheat, rice or barley R2R3 MYB genes are differentially expressed by abiotic stresses such as salt, cold, heat, boron or abscisic acid (ABA) treatment (Katiyar et al., 2012; Zhang et al., 2012; Tombuloglu et al., 2013; Zhao et al., 2017). Further, overexpression of some of these MYB genes in Arabidopsis or tobacco can confer abiotic stress tolerance (Zhang et al., 2014; He et al., 2012; Zhao et al., 2017; Wei et al., 2017a).

Relatively few studies have looked at the effects of modulating MYB gene expression in crop plants such as barley. In this work the role of the first barley MYB gene to be described, *HvMYB1* (originally termed *MYBHv1*) (Marocco et al., 1989; Wissenbach et al., 1993), has been investigated. A wheat homologue, *TaMYB1*, was previously shown to be upregulated by hypoxia and salt stress (Lee et al., 2007), and another wheat orthologue (*TaMYB1D*) can enhance drought and oxidative stress when expressed in the dicot tobacco (Wei et al., 2017b). In this work the expression of *HvMYB1* in barley plants (*Hordeum vulgare* L.), and the effects of modifying its gene expression levels in transgenic barley plants on drought stress were investigated.

2. Materials and methods

2.1. Barley cultivar and growth conditions

The barley cultivar Golden Promise was used to provide immature embryos for transformation. Plants for donor embryos and for drought stress tests were grown in an environmentally controlled growth chamber at 18 °C with a 16 h photoperiod (light level of 450 μE m − 2 s − 1), and a relative humidity between 60 and 80%. The seeds were planted in 8 cm pots. At the time of planting, 1 g of slow release fertiliser (Osmocoat N:P:K = 14:13:13) was put onto the surface of the soil. Fourteen-day-old transgenic and wildtype plants grown as above in triplicate were subjected to drought stress by continuous water deprivation for 14 days (mean loss of 78.5% weight from soil at field capacity), watering on alternate days in the case of controls. Root and leaf tissues were harvested, frozen in liquid nitrogen and stored at − 80 °C until further analysis.

2.2. Hydroponic treatments

Wildtype Golden Promise grains were transferred to moistened filter paper for stratification at 4 °C for 24 h and then placed in an environmentally controlled growth chamber to germinate. 5 day old uniform seedlings were then transplanted into a hydroponic system in a modified Hoagland solution based on Hoffmann et al. (2012). 14 days after transplanting, 100 μM ABA, or 20% (w/v) polyethylene glycol 6000 (PEG) were added to induce stress (Cardi et al., 2011; Liu et al., 2008). A salt treatment was performed according to Cao et al. (2016) with a final concentration of 150 mM NaCl added. A supplement of 3.8 mM $CaCl_2$ was added to the nutrient solution to maintain free Ca2 + levels for salt treated plants. For all treatments, root and leaf tissues were harvested after 2 and 7 days, frozen in liquid nitrogen and stored at − 80 °C until further analysis. The solutions were continuously aerated with an air pump. The pH in each container was adjusted once a day with 1 M HCl or NaOH as required.

2.3. Plasmid construction and transformation

A full-length open reading frame for *HvMYB1* (Genebank accession number X70877) was PCR-amplified from Golden Promise with primers HvMYB1-F (AGCTCTAGAACCATCTAAAGCGATGG) and HvMYB1-R (AGCTCTAGATGCTCATTTCATCTCGATG) (introduced *Xba*1 sites are underlined) using cDNA from Golden Promise leaves as template and cloned in the sense and antisense orientation into the *Xba*1 site between an actin promoter and OCS terminator contained within the binary vector pWBVec8 which harbours a hygromycin resistance gene cassette (Wang et al., 1997; Abass and Morris, 2013). Clones were checked by sequencing before introducing the constructs into *Agrobacterium tumefaciens* strain EHA105. For transformation the method described by Tingay et al. (1997) was followed. Two weeks after pollination spikes of healthy plants were collected, immature embryos were excised and inoculated with *A. tumefaciens*. Callus induction and regeneration of barley plants was performed under hygromycin selection.

For intracellular localisation studies, the open reading frame of *HvMYB1* was transcriptionally fused to the 5' end of *GFP* from pCambia 1302, between the CaMV 35S promoter and the Nos terminator, introduced into *Agrobacterium tumefaciens* and used for transient transformation of onion epidermal cells (Sun et al., 2007).

2.4. RNA extraction and mRNA expression

Total RNA was isolated from leaves and roots using TRIzol Reagent (Life Technologies, Rockville, MD). DNA was removed with RQ1 DNase (Promega). Two μg of total RNA was used to make cDNA using I script cDNA Synthesis Kit (BioRad) as per the manufacturer's instructions. Following synthesis, cDNA was diluted to 1/10. qRT-PCRs were performed in triplicate with Absolute SYBR green mix (ThermoFisher scientific) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The expression of the genes *HvMYB1, HvA1, CATALASE1 (HvCAT1), CATALASE2 (HvCAT2), ASCORBATE PEROXIDASE1 (HvAPX1), ASCORBATE PEROXIDASE2 (HvAPX2), GLUTATHIONE PEROXIDASE1 (HvGPX1), GLUTATHIONE PEROXIDASE2 (HvGPX2), DEHYDRIN RESPONSE ELEMENT 1 (HvDREB1), ABA RESPONSIVE BINDING FACTOR 2 (HvABF2), DEHYDRIN 6 (HvDHN6),* and *ABSCISIC ACID INSENSITIVE 5 (HvABI5)* was measured. Oligonucleotides were designed using Primer3plus (Untergasser et al., 2007) other than for *HvDHN6* (Qian et al., 2008) and are shown in Table 1. Standard cycling conditions

Table 1
QPCR primers.

Gene Name	Forward	Reverse	Amplicon bp	Genbank
HvCAT1	CCCGTCTGGAACAACAAC	CCCCGTGCATGAACAAC	134	U20777
HvCAT2	CGACGACAAGATGCTGCAGT	TGGTTGTTCTTGAAGCCGC	122	U20778
HvAPX1	CGGAGCTTTTGAGTGGTGACA	CCGCAGCATATTTCTCCACAA	107	AJ006358
HvAPX2	CGCCGAGAAGAACTGC	GCCGGTCTTGGTGGC	82	AF411228
HvGPX1	AACGGCAACAATGTTTCTCC	ACAACGTGACCCTCCTTGTC	119	AJ238745
HvGPX2	ACGTGAATGGCAACAATGCT	ATGACATGCCCCTCTTTGTC	124	AJ238744
HvMYB1	ACCACGTGGACCACCATCAC	TGCACAGGTCCAGGTTCAGG	98	X70877
HvA1	ACAAGCAGTCGATCCATTCC	CTCAAACAAACACGAACTGGAA	83	AK358283
HvADP	GCTCTCCAACAACATTGC	GAGACATCCAGCATCATTCATTCC	77	AJ508228
HvDREB1	TGTCTCAAATGCGCCAACAG	TTTCACCTTATGCGCCACAG	88	DQ012941
HvABI5	AGGAGTCAACATCGACATTCCG	AGCAGCACGAGAAAGGAAAC	122	AK373571
HvABF2	AGAGGCGCATGATCAAGAAC	AGTTTTGCTACCTCGGCTTC	94	AK363330
HvDHN6	TTTTACCGTGTGATAGATGTTGCA	TGCAAACCGACCAGACAAACT	72	AF043091

were 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C; then the melting curve profiles were determined. Gene expression values were normalised to *HvADP* due to its stability of expression under drought conditions (Ferdous et al., 2015) and gene expression values under drought stress relative to control well-watered conditions were calculated using the $2^{-\Delta\Delta CT}$ (Schmittgen and Livak, 2008).

2.5. Physiological measurements

For Relative Water Content (RWC), leaves and roots were harvested and the fresh weight (FW) of each was measured. Plant material was then placed in 50 ml tubes filled with water, and kept overnight in darkness at 5 °C. The turgid weight (TW) was then measured. The plant material was then dried at 80 °C for 48 h and the dry weight (DW) measured. RWC was calculated using the following formula: RWC = FW-DW/TW-DW x 100.

For leaf Water Loss Rate (WLR), leaves were removed from unstressed barley seedlings, weighed and placed on filter paper in a Petri dish at room temperature (21 °C). Leaves were weighed every 60 min until the decrease in weight stopped or slowed significantly (up to a total of 360 min). Samples were dried at 80 °C for 48 h and the dry weight measured. LWR in g/h per g dry weight was calculated using the following formula: WLR = (FWT1 − FWT2)/[DW × (T2 − T1)]. FWT1 is leaf fresh mass at time T1, FWT2 is leaf fresh mass at time T2, DW is leaf dry weight, T1 is time for FWT1, and T2 is time for FWT2. Stomatal conductance was measured using an AP4 cycling porometer (Delta-T Devices Ltd, Cambridge, UK) based on the methods described by Prats et al. (2006). Leaf measurements were taken from 3 plants of each line, under both control and drought conditions described above. Measurement of free proline content was carried out according to Bates et al. (1973). Catalase activity (EC 1.11.1.6) was assayed according to the methods described by Roy et al. (2009). Hydrogen peroxide was measured as described by Jana and Choudhuri (1982). Data was analysed for statistical significance by ANOVA and post-hoc Tukey's test for multiple comparisons.

3. Results

3.1. HvMYB1 gene expression and protein localisation

Expression of *HvMYB1* and the drought-induced gene *HvA1* (Hong et al., 1992) was measured in roots and leaves of wildtype barley plants grown for 14 days and exposed to a further 14 days of drought. Control plants were grown for 28 days under well-watered conditions. Elevated *HvMYB1* transcript levels were observed in both roots and leaves after drought, in a similar manner as seen for *HvA1*, with higher levels observed in roots compared to leaves (Fig. 1A). Transient expression in onion epidermal cells of HvMYB1 translationally fused to GFP showed the fusion protein to be both cytosolic and nuclear located (Fig. 1B), which is consistent with the score of 5 for a nuclear localisation signal when using the cNLS Mapper tool (Kosugi et al., 2009). To further characterise *HvMYB1* gene expression, wildtype barley plants were grown in hydroponic solution and stressed by the addition of ABA, NaCl or polyethylene glycol. Little change in gene expression for *HvMYB1* or for *HvA1* was seen after 2 days (Fig. 1C) and after 7 days ABA and NaCl had a minor effect on *HvMYB1* expression, however PEG treatment was most effective in stimulating *HvMYB1* expression (13-fold relative to the unstressed controls). In contrast, *HvA1* expression was strongly enhanced by ABA, NaCl and polyethylene glycol (Fig. 1D).

To test whether *HvMYB1* can influence tolerance to drought stress, overexpression and antisense constructs with the *HvMYB1* coding region under the control of the actin promoter were introduced into barley cv. Golden Promise. 8 independent primary overexpresser and 3 antisense lines were recovered, based on hygromycin selection and PCR analysis of primary transformants. The antisense lines did not flourish, produced non-viable seed, and were not studied further. Three *HvMYB1* overexpresser lines were then chosen for biochemical and physiological measurements of drought tolerance. *HvMYB1* expression levels in these lines was measured in homozygous plants grown under well-watered conditions, and also in a transgenic empty vector line. The overexpresser lines OX1, OX2 and OX3 showed enhanced expression of mRNA for *HvMYB1* 15 to 20 times more abundant compared to the wildtype and empty vector control plants (Fig. 1E). Using PlantCARE software (http://bioinformatics.psb.ugent.be/webtools/plant care/html/), the promotor region (1000bp upstream of the 5′UTR) of *HvMYB1* was searched for consensus sequences for transcription factor binding; an ABA response element (ABRE) is present 740 bp upstream from the transcription start point.

3.2. Drought stress resistance

To test the drought response in *HvMYB1* overexpresser lines, homozygous plants from three lines were grown for 14 days under well-watered conditions, then water withheld for a further period of 14 days. Control plants were grown for 28 days under well-watered conditions. The wildtype plants had begun wilting and turning yellow after the 14 day drought period, whilst the overexpresser lines were more robust and showed no yellow colouring or wilting. Control and drought plants were assayed for RWC which was close to 90% in the roots and leaves of all the control (both wildtype and transgenic) plants. There was a drop in the RWC for both roots and leaves in the wildtype and overexpresser lines following drought, however the reduction was less in the overexpresser lines compared to the wildtype (Fig. 2 A and B). Consistent with the higher leaf RWC observed in the overexpresser lines, leaf water loss rate was lower in overexpressor lines compared to the wildtype plants (Fig. 2 C). The effect of *HvMYB1* overexpression on stomatal conductance was then measured. Following 14 days of drought, stomatal conductance was significantly lower in all of the transgenic overexpresser lines compared to the wildtype Golden Promise (Fig. 2D). No differences in stomatal conductance were found between any of the lines under well-watered conditions, and there was no significant difference between overexpresser lines1-3 and the wildtype in stomatal density (stomata per mm^2 and standard deviation: WT 58.5 ± 12.26, OX1 55.5 ± 11.17, OX2 44 ± 8.44, OX3 47.5 ± 16.2).

Plants were assayed for key biochemical parameters associated with plant drought. The osmolyte proline content was measured in both roots and leaves. Under the control well-watered conditions there was no difference in proline content between the wildtype and overexpresser lines in either roots or leaves. In wildtype plants, drought caused proline levels to increase in both roots and leaves but not with any statistical significance. In contrast there was a significant increase in proline content observed in the overexpresser lines in comparison to the wildtype (Fig. 3 A and B).

Levels of the reactive oxygen species hydrogen peroxide (H_2O_2) in wildtype and overexpresser lines were very similar in roots and leaves prior to drought stress. In leaf material of the wildtype plants there was a significant increase after 14 days of drought. There was also an increase observed in the overexpresser lines, but this was significantly less in lines 1 and 2 than for the wildtype. A similar trend was seen in the roots of the plants (Fig. 4 A and B). Activity of catalase, an enzyme responsible for regulating levels of reactive oxygen species by breaking down H_2O_2, was also measured. Catalase activity (EC 1.11.1.6) was similar for the wildtype and overexpresser lines in roots prior to the imposition of drought, but was constitutively lower in leaves. There was a significant increase in catalase activity in the leaves of control plants following drought, but this was not observed in the overexpresser lines. In roots, catalase activity increased by almost 100-fold in the control plants while no significant increase was detected in the overexpresser lines (Fig. 4 C and D).

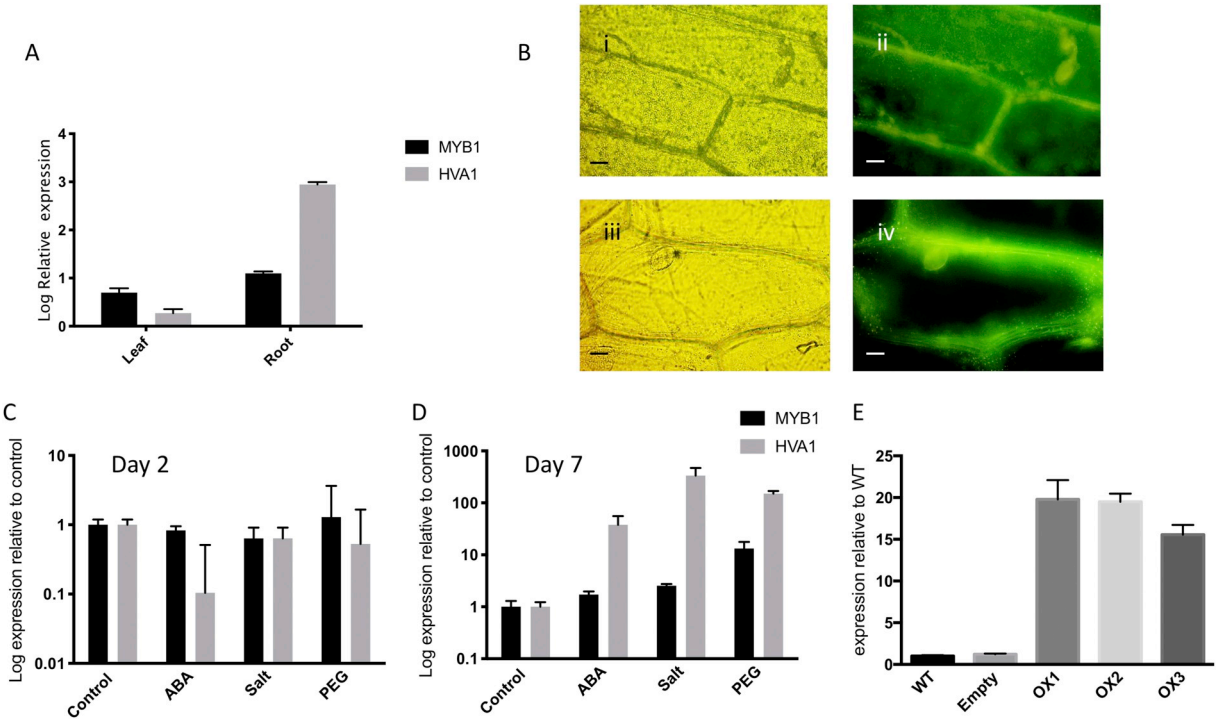

Fig. 1. (A) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *HvMYB1* and *HvA1* in leaves and roots of wildtype Golden Promise after 2 weeks of drought. Transcript levels were normalised with respect to the reference gene *HvADP* and are presented relative to control well-watered conditions. (B) Transient expression in onion epidermal cells. (i) free GFP brightfield, (ii) free GFP, (iii) HvMYB1-GFP brightfield, (iv) HvMYB-GFP. The size bar indicates 50 μm. (C) qRT-PCR analysis of *HvMYB1* and *HvA1* transcripts in response to abiotic stress (100 μM abscisic acid (ABA), 150 mM NaCl, 20% w/v polyethylene glycol (PEG)) in hydroponic culture after 2 days and (D) 7 days. (E) qRT-PCR analysis of *HvMYB1* transcript expression in wildtype and transgenic (empty vector, overexpresser OX1, OX2 and OX3) barley lines. Transcript levels are presented relative to the reference gene *HvADP*. Values are mean ± SD of three replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

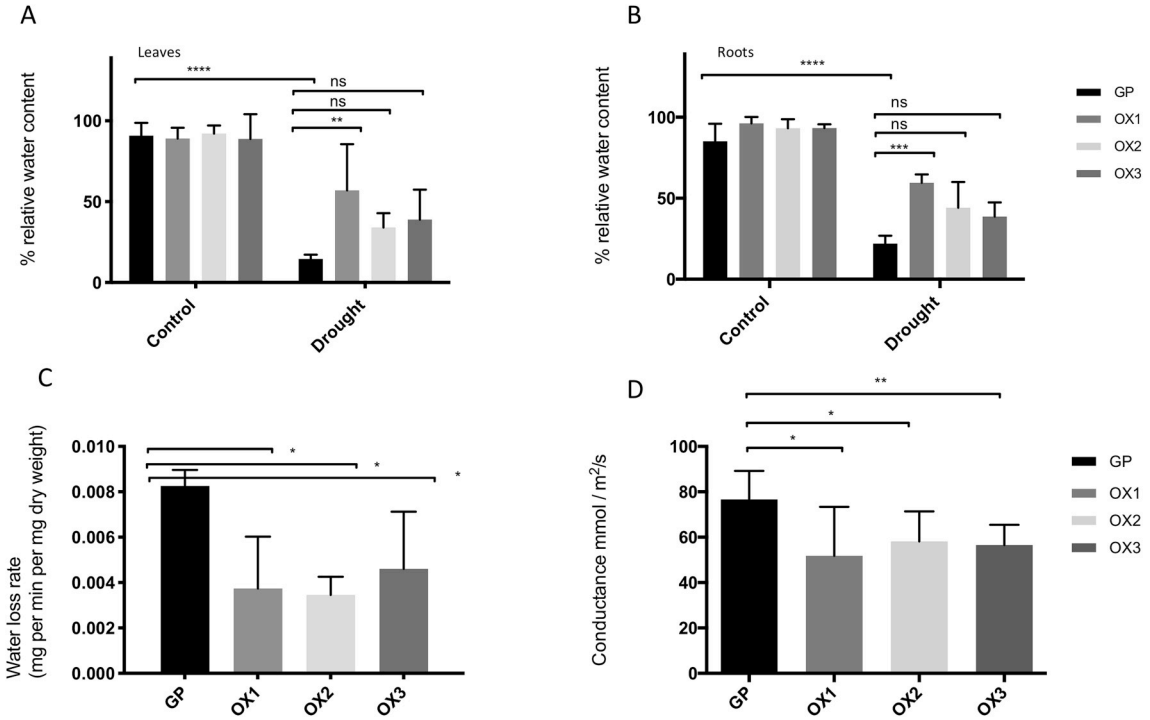

Fig. 2. The effect of *HvMYB1* overexpression on water loss and retention under control conditions or 14 days drought. (A and B), relative water content of leaves and roots respectively of wildtype (GP) and overexpressing (OX1, OX2 and OX3) barley lines. (C) Leaf water loss rate of wildtype (GP) and overexpressing (OX1, OX2 and OX3) barley lines. (D), Effect of *HvMYB1* overexpression (OX1, OX2 and OX3) on stomatal conductance after 14 days drought. Values are means ± SD. ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, and ns no significance.

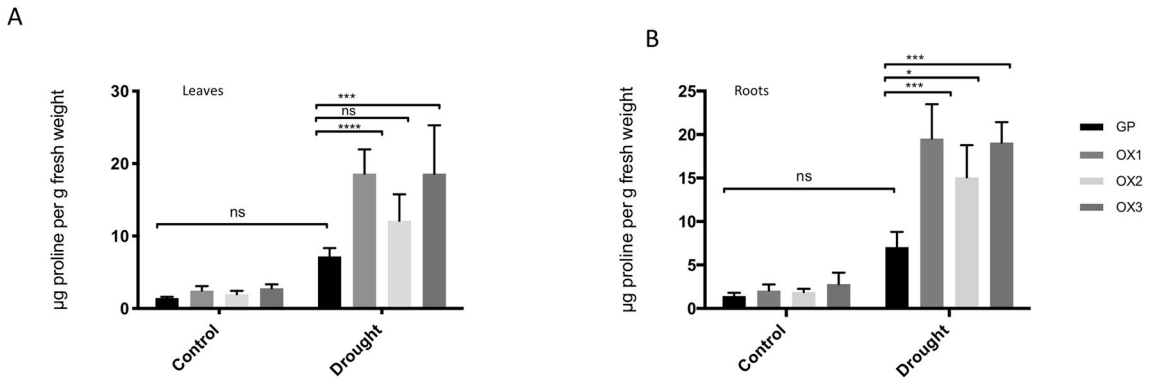

Fig. 3. The effect of *HvMYB1* over-expression on proline content under control conditions or 14 days drought. Proline content in leaves (A) and roots (B) of wildtype (GP) and transgenic over expressing (OX1, OX2 and OX3) barley lines. Values are means ± SD. ****P < 0.0001, ***P < 0.001, *P < 0.05, and ns no significance.

3.3. Abiotic stress gene expression

A number of abiotic stress responsive genes were chosen for further analysis of the role of HvMYB1 in drought stress. HvDREB1 is a transcription factor induced by abiotic stress and ABA (Xu et al., 2009), HvABI5 is a transcription factor that is a key component of ABA signalling (Casaretto and Ho, 2003), as is HvABF2 (Choi et al., 2000). HvDHN6 is a dehydrin LEA protein that is upregulated in response to ABA (Kosová et al., 2014). Gene expression in leaves before and after drought imposition was determined by qRT-PCR analysis of RNA from leaves. Prior to drought imposition, leaf *HvDREB1* expression was slightly lower in the *HvMYB1* overexpressing lines as was *HvABF2*. *HvABI5* and *HvDHN6* expression was notably lower in the *HvMYB1* overexpressing lines (Fig. 5A). Expression of the three transcription factor genes was strongly enhanced by drought in both wildtype and transgenic lines, as was *HvDHN6* expression, however this was higher in the *HvMYB1* overexpressing lines than in the wildtype (Fig. 5B). The promotor regions (1000 bp upstream of the 5′UTR) of all genes were analysed for the presence of MYB binding cis-elements, which revealed that the promoters of all four genes contain multiple MYB recognition and binding sites.

3.4. ROS scavenger gene expression

Gene expression for two catalase genes, for two ascorbate peroxidase genes and for two glutathione peroxidase genes was measured in well-watered and in droughted leaves of wildtype and the three *HvMYB1* overexpresser lines. These enzymes are involved in the breakdown of hydrogen peroxide at different degrees of oxidative stress. Prior to drought imposition, *CAT1* and *CAT2* gene expression did not differ between control and transgenic lines, however all three *HvMYB1* overexpresser lines had constitutively higher levels of *APX1*, *APX2*, *GPX1* and *GPX2*, under non-stress conditions (Fig. 6A). The levels of ROS scavenger gene expression in droughted HvMYB1 overexpresser lines were closer to the levels seen in the wildtype after drought, with the exception of *GPX2* in line OX1 (Fig. 6B). Promoter sequence analysis revealed that the promoters of all six genes contain multiple MYB recognition and binding sites.

4. Discussion

Gene expression patterns for the barley *HvMYB1* gene were initially analysed in transgenic tobacco plants using promoter-*GUS* gene fusions,

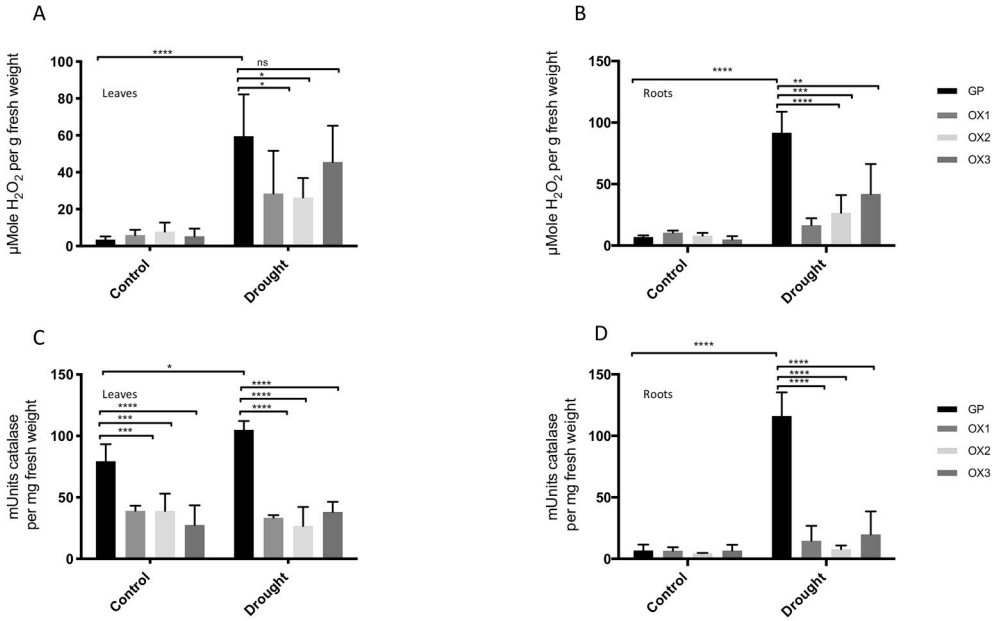

Fig. 4. (A) The effect of *HvMYB1* over-expression (OX) on barley reactive oxygen systems under control or 14 days drought. Hydrogen peroxide levels in wildtype (GP) and transgenic barley (OX1, OX2 and OX3) leaves (A) and roots (B). Catalase enzyme activity levels in control and transgenic barley leaves (C) and roots (D). Values are means ± SD. ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, and ns no significance.

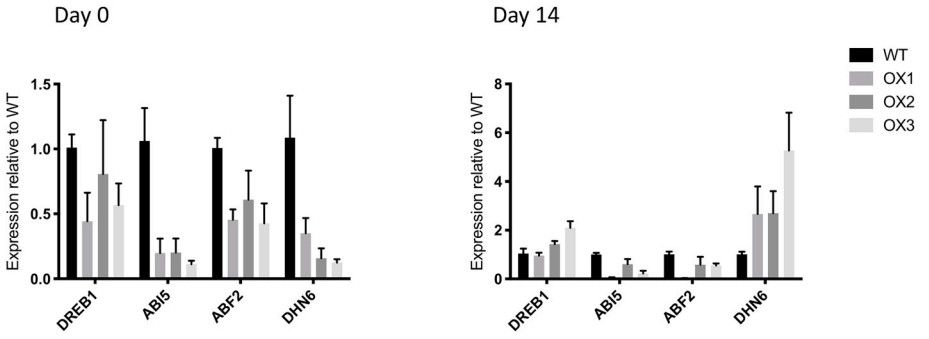

Fig. 5. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of abiotic stress induced genes (*HvDREB1, HvABI5, HvABF2, HvDHN6*) in leaves of wildtype and *HVMYB1* overexpressing (OX1, OX2 and OX3) barley lines grown under (A) control conditions for 14 days and (B) imposition of a further 14 days drought. Transcript levels were normalised with respect to the reference gene *HvADP*. Values are mean ± SD of three replicates.

from which it was inferred that this transcription factor was involved as a regulator of meristematic activity (Wissenbach et al., 1993). Later studies on the wheat orthologue *TaMYB1* showed that this gene is upregulated by abiotic stress in wheat roots (Lee et al., 2007), and that overexpression of the wheat paralogue *TaMYB1D* in tobacco enhanced drought and oxidative stress resistance (Wei et al., 2017b). The data presented here also indicates that HvMYB1 is involved in regulating abiotic stress, as expression levels of *HVMYB1* were enhanced after imposition of drought in both roots and leaves in a similar manner to a previously characterised drought-induced barley gene (*HvA1*). Osmotic stress (PEG) also induced *HVMYB1* expression but both salt stress and ABA were less effective in doing so. In order to investigate the role of barley HvMYB1 in the native background, transgenic barley plants were produced and analysed. Antisense lines did not produce viable grains, thus it was not possible to analyse these further, but does suggest a critical role of HvMYB1 in plant growth and fertility.

Overexpression lines with up to twenty-fold enhanced expression of *HvMYB1* were viable and homozygous lines were identified and studied for their response to drought. When compared to wildtype barley, the overexpresser lines showed clear physiological traits for drought tolerance; relative water content of roots and leaves was enhanced under drought conditions, water loss rates were reduced and stomatal conductance was lower under drought, indicating that the overexpressers were more able to retain water and thus mitigate against the effects of drought. *HvMYB1* overexpressers also displayed a molecular phenotype consistent with constitutive drought stress tolerance. Dehydrins are late embryogenesis abundant proteins thought to play a protective function in abiotic stress, and under control conditions, *HvDHN6* was slightly downregulated by *HvMYB1* overexpression, but strongly upregulated relative to wildtype plants under drought conditions.

The function of HvMYB1 resembles that of Arabidopsis AtMYB44. This gene is upregulated by abiotic stress and overexpression results in enhanced drought tolerance and reduced water loss (Jung et al., 2008). AtMYB44 has been demonstrated to interact with the cytosolic and nuclear located ABA receptor RCAR1/PYL9, however it is not clear how the reported competition of AtMYB44 with ABI1 for binding to RCAR1/PYL9 would lead to the drought tolerance phenotype seen in the AtMYB44 overexpression lines (Li et al., 2014), since the RCAR1/PYL9 – ABI1 interaction is known to inhibit the phosphatase activity of ABI1 and thus promote SNRK2 activation of ABA responsive gene expression (Raghavendra et al., 2010). In contrast to both HvMYB1 and AtMYB44, Arabidopsis AtMYB60 is a negative regulator of stomatal opening and gene expression. It has been shown to be downregulated by drought, and exogenous ABA and null mutants are more drought resistant (Cominelli et al., 2005). HvMYB1 may therefore have an opposite function to AtMYB60, since many of the traits for overexpression of *HvMYB1* are similar to those for loss of function *AtMYB60*.

MYB transcription factors are also involved in other aspects of ABA signalling, for example gene expression of the transcription factor ABI5, one of the main regulators of ABA-induced gene expression particularly during germination and seedling growth, is itself regulated by MYB proteins. AtMYB96 is a positive regulator and AtMYB7 is a negative regulator of ABI5 gene expression in Arabidopsis (reviewed Skubacz et al., 2016). The data shown here indicates that HvMYB1 acts as a negative regulator of *HvABI5* expression, suggesting that HvMYB1 may be primarily involved in stress responses at the post-germination stage.

Water can be conserved in the plant by preventing loss through the stomata, but also by physically retaining water by enhancing the levels of cellular osmolytes such as proline. Proline synthesis is thought to be driven by the ABA-regulated enzyme P5CS (Bandurska et al., 2017). The *HvMYB1* overexpresser lines had significantly higher levels of proline under drought conditions, which would contribute to their drought tolerant phenotype, and is consistent with a phenotype that suggest an upregulated response to ABA.

Drought damage to plants is caused in part by oxidative stress; photorespiration occurs in C3 plants due to reduced CO_2 in the leaf results in the production of reactive oxygen species (ROS) such as hydrogen peroxide. Reduced ROS levels under stress are associated with drought tolerance in barley (Marok et al., 2013). Plants mitigate against oxidative stress by breaking down ROS through enzymes such as ascorbate peroxidase and glutathione peroxidase, which are in the first line of defence against ROS. Higher levels of ROS are removed by catalase; higher activity levels of catalase under stress conditions are associated with drought tolerance (Simova-Stoilova et al., 2008). Both

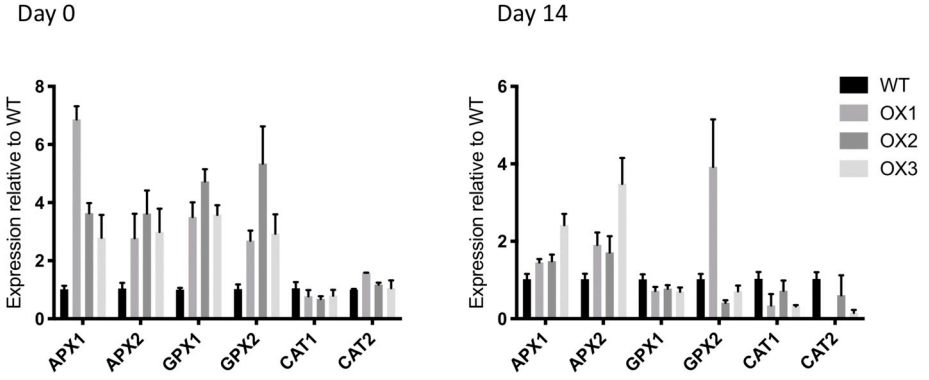

Fig. 6. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of oxidative stress associated genes (*APX1, APX2, GPX1, GPX2, CAT1, CAT2*) in leaves of wildtype and *HVMYB1* overexpressing (OX1, OX2 and OX3) barley lines grown under (A) control conditions for 14 days and (B) imposition of a further 14 days drought. Transcript levels were normalised with respect to the reference gene *HvADP*. Values are mean ± SD of three replicates.

leaves and roots of the overexpresser lines under drought stress did not increase hydrogen peroxide levels to the same degree as the wildtype, and this was reflected by the lower catalase activity of the plants., catalase activity was strongly enhanced by drought in both leaves and roots of wildtype plants, but not in the overexpresser plants, possibly a consequence of the lower levels of ROS in the overexpressers. The wheat MYB *TaODERANT1*, by contrast, when overexpressed in tobacco, gave higher levels of catalase enzyme activity after drought treatment (Wei et al., 2017a).

CAT gene expression in unstressed *HvMYB1* overexpressor plants was not notably different from that of wildtype plants however the expression levels of *APX* and for *GPX* was constitutively higher in unstressed *HvMYB1* overexpresser plants. Catalase, which has a lower affinity for H_2O_2 than *APX*, plays a major role in severe drought stress, whereas the ascorbate and glutathione cycle are of more importance during moderate stress, with GPX and APX considered as a first line of defence against ROS (reviewed de Carvalho, 2008; Gill and Tuteja, 2010). Expression of ROS scavenging enzymes are thought to be regulated by ROS production (Guan et al., 2000). Thus, the constitutively high levels of *APX* and *GPX* in the overexpresser *HvMYB1* plants may be sufficient to hold drought stress in check such that enhanced *CAT* levels are not required. After imposing drought stress, ROS scavenging gene expression levels were more in line with the wildtype levels, again indicative of a protective role for pre-emptive ROS scavenging gene expression.

The evidence presented here is consistent with a primary role for HvMYB1 in regulating drought stress in barley; the gene is upregulated by drought stress in both roots and leaves, overexpression of *HvMYB1* results in plants that show enhanced water retention under drought stress through a combination of reduced stomatal conductance and enhanced proline production, and oxidative stress-causing ROS accumulation is reduced through constitutively higher levels of the ROS scavengers such as APX and GPX.

Contributions

Charlotte Wendelboe-Nelson carried out experimental work. Ross Alexander carried out experimental work and wrote the manuscript. Peter Morris led the work, carried out experimental work and wrote the manuscript.

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