The response mechanism of the *HVA1* gene in hulless barley under drought stress

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Abstract

HVA1, a member of LEA3 (late embryogenesis abundant protein, group 3), is closely related to water stress. However, the response of *HVA1* to drought remains unknown in hulless barley. In this study, cultivars with high (Handizi), intermediate (Kunlun 12), and low (Dama) drought tolerance were selected from 28 hulless barley cultivars from the Tibet-Qinghai plateau to explore the drought response mechanism of *HVA1*. Then, *HVA1* was cloned and the expression of the three cultivars was studied using exposure to polyethylene glycol (PEG) 6000. *HVA1*s in the three hulless barleys were highly homologous at the nucleotide and amino acid levels with over 99% identity. Real-time quantitative polymerase chain reaction showed that the expression level of *HVA1* induced by PEG 6000 had a single peak curve in the three cultivars, but higher *HVA1* transcript accumulation was seen in Handizi than in Kunlun 12 and Dama under the same drought stress. This result was also proved in eight hulless barleys. The expression level was a better predictor of drought resistance than the genetic structure of *HVA1*.

Introduction

Hulless barley (*Hordeum vulgare* L. *var. nudum* Hook. f.) is a selfing annual species, with naked grains when ripening. It is widely grown on the Qinghai-Tibet plateau (suffering serious drought stress) and has been a staple food for the Tibetan people since the fifth century CE (Liang *et al*., 2012). Drought is an important environmental constraint that limits the productivity of barley and other crops worldwide (Romanek *et al*., 2011). The growth and development of plants are restrained under the stress of drought with decreases in net photosynthetic rate, respiration, leaf osmoregulation ability, and cell membrane stability (Li *et al*., 2016). The most susceptible stages to drought are germination, early seedling growth, and grain filling. If plants can survive drought stress during these sensitive periods, the ability of the plant to survive additional drought exposure will increase (Liang *et al*., 2016).

Plants have developed many physiological and biochemical reactions in response to adverse environmental conditions. Some compatible low-molecular-weight metabolites will be accumulated to protect cells against dehydration, and the most common of these is late embryogenesis abundant protein (LEA) (Park *et al*., 2003). LEA proteins are closely related to desiccation induced and regulated by abscisic acid (ABA) or dehydration signaling (Ramanjulu and Bartels, 2002). LEA proteins are involved in protection mechanisms against environmental stressors in plants (Liang *et al*., 2013). According to the homology of the amino acid sequence and presence of special primitive sequences, LEA proteins are categorised into six groups (Wise, 2003). The *Hordeum vulgare aleurone1* (*HVA1*) gene, which belongs to group 3 LEA, is activated during cell dehydration caused by water deficit, salt stress, low temperature, or ABA induction (Romanek *et al*., 2011; Battaglia *et al*., 2008).

Most researches on the *HVA1* have focused on transformation. The *HVA1* plays a protective role against water tolerance in rice (Babu *et al*., 2004), wheat (Chauhan and Khurana, 2011), oats (Oraby *et al*., 2005), tobacco (Li *et al*., 2007) and mulberry (Checker *et al*., 2012). *HVA1* resists water stress by increasing the dry weight of plant, the fresh weight of the roots, and the dry weight of the shoots in transgenic wheat (Sivamani *et al*., 2000). Crops transformed by the introduction of the barley *HVA1* had a significant increase in vegetative biomass and other traits associated with drought tolerance. However, the expression pattern and the mechanism of *HVA1* under drought stress in hulless barley remain unknown.

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Materials and methods

Plant materials and growth conditions

The 28 hulless barley cultivars were selected from 300 cultivars, which collected from several major planting provinces including Qinghai, Tibet, and Sichuan. Selection was based on high yield and disease-resistance (Table 1). The cultivars were divided into three groups based on geographic origin: Qinghai-

Tibet Plateau (19 cultivars), Hunan and Jiangsu provinces of China (5 cultivars), and Mexico (4 cultivars). The cultivars were archived by the Qinghai Academy of Agricultural Forestry Sciences. Seeds were grown in 100 mm petri dishes with three layers of filter paper saturated with 10 mL of distilled water for 7 d with sufficient additional water added to maintain saturation. Seedlings were transplanted into a 100 mL breaker (10 plants per breaker) with 20 mL distilled water. The seedlings were maintained in an incubator at 25°C with 2000 lx lighting intensity and a 14 h:10 h light:dark photoperiod.

Detection of relative water content and relative water loss rate

When the third leaves of 28 hulless barley varieties were fully expanded (15 d after sowing), they were removed to measure the relative water content and dehydration rate according to a previously described protocol (Chapotin *et al*., 2003). We used the following formula: Relative water content (RWC) (%)=[(Fresh quality–Dry quality)/(Saturated quality–Dry quality)] × 100%; Relative water loss rate (RWL) ($g \cdot g^{-1}DW \cdot h^{-1}$)=(Fresh quality–quality after 24 h dehydration)/(Dry quality × 24).

Polyethylene glycol 6000 treatment

Based on the RWC and RWL results, Handizi, Kunlun 12, and Dama were selected for further experiments. These three cultivars were treated with different concentrations (0, 5, 10, 15, 20, 25, and 30%) of polyethylene glycol (PEG) 6000 (Sigma Aldrich, Saint Louis, MO, USA) at the three-leaf stage in an incubator at 25ºC with 2000 lx lighting intensity and a 14h:10h light:dark photoperiod. Sufficient PEG 6000 solution was added to each dish every day so that simulated drought conditions could be maintained for 3 d. Then the relative conductivity and malondialdehyde content of leaves were measured according to Karami *et al*. (2013), while the soluble protein content of leaves was determined according to Bradford (1976).

Isolation of the *HVA1*

Total RNA was extracted from the leaf tissues of Handizi, Kunlun 12 and Dama using MiniBEST Plant RNA Extraction Kit (TaKaRa, Kusatsu, Japan). The integrity of RNA was determined by electrophoresis on a 1.0% formaldehyde-denatured agarose gel stained with Gold View. The quality and quantity of RNA was determined by measuring the $OD_{260/280}$ and OD_{260} with a SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA). The cDNA was synthesised using Superscript First-Strand Synthesis System for RT-PCR (TaKaRa) and an adaptor-oligo (dT) primer following manufacturer instructions. Primers were designed and used to amplify the cDNA of the *HVA1*gene (Table 2). Primers (P1) were designed to amplicate *HVA1* according to the sequence in GenBank (ID: X78205.1). The PCR amplification conditions were 94°C for 5 min followed by 30 cycles at 94°C for 1 min; 64°C for 40 s; 72°C for 1 min and a final 72°C for 8 min. The PCR products were cloned into the pMD20-T vector (TaKaRa), and then transformed into *E. coli* DH5α. Five positive clones were sent for sequencing.

Semi-quantitative polymerase chain reaction and quantitative real-time polymerase chain reaction

Total RNA was extracted from the leaves of Handizi, Kunlun 12 and Dama which were soaked in 0 to 30% PEG 6000 for 3 d. The total RNA was then reverse transcribed into cDNA as a template for PCR. The semi-quantitative PCR primers of *HVA1* were P1. Primers (P2) of reference gene β-actin were designed according to barley actin (ID: AY145541). The cycling parameters of semi-quantitative PCR amplification were: 95°C for 5 min followed by 32 cycles at 94°C for 60 s, 64°C for 60 s, 72°C for 90 s and a final 72°C for 10 min. The quantitative real-time PCR primers (P3) were designed according to the *HVA1* and the primers (P4) of the reference gene were designed from 18S rRNA. The qPCR amplification conditions were 95°Cfor 3 min followed by 40 cycles at 95°C for 10 s, 61°C for 30 s, then 95°C for 1 min, 61°C for1 min and a final 40 cycles at 61°C for 10s. The fluorescence signals obtained were measured once for each cycle at the extension step. All the reactions were performed in a DNA Engine Opticon™ 2 system (Bio-Rad) following manufacturer recommendations.

Data analysis

All physiological and gene expression measurements were replicated at least three times with independent plant samples and the mean was used for result analysis and discussion. Means, standard deviation (SD) and statistical analysis were performed using SPSS package (version 18.0). All data were subjected to variance analy-

Table 1. Names and sources of the hulless barley cultivars used in this study.

Number	Name	Source	Number	Name	Source
1	Dama	Gansu	15	GolasCley	Mexico
2	Ganziheiliuleng	Sichuan	16	Xiang 84-26-174	Hunan
3	Sunong 401	Jiangsu	17	Zangqing 80	Xizang
4	Xiang 1146	Hunan	18	Sunjiazhuangbai	Gansu
5	Crime	Mexico	19	Minxian	Sichuan
6	9748	Gansu	20	Dulihuang	Gansu
7	Daimao	Sichuan	21	Changshengzi	Gansu
8	Aba 330	Sichuan	22	Dagestam	Mexico
9	Kunlun 10	Xining	23	Rudong 4	Jiangsu
10	Beiqing 1	Qinghai	24	Beiqing 3	Qinghai
11	Lasagoumang	Xizang	25	Gaoyuanzao 1	Qinghai
12	Kunlun 12	Xining	26	Hor1726	Mexico
13	Kangqing 7	Sichuan	27	Liulengtou	Gansu
14	Xiang 0888	Hunan	28	Handizi	Sichuan

sis (ANOVA) and the mean differences were compared by the least significant difference (LSD) test. Nucleotides and amino acid sequence analyses were performed with the DNAMAN program version 5. 2. 2 (Lynnon Corp., San Ramon, CA, USA). $2^{-\Delta\Delta CT}$ methods were used in quantifying the relative changes of gene expression (Pfaffl, 2001).

Results

Identification of drought resistance in hulless barley cultivars

The RWC and RWL results of the 28 cultivars showed that the relative water content was the highest (60.16%) and the relative water loss rate was the lowest (8.80%) in Handizi, and the relative water content was the lowest (38.98%) and the dehydration rate was the highest (20.20%) in Dama (Figure 1A). These two parameters in Kunlun 12 were intermediate. The results suggested that Dama was the most sensitive to water loss stress and Handizi was the least sensitive, with Kunlun 12 being intermediate. Dama, Kunlun 12 and Handizi were selected for the next experiments.

We detected the RWC and ABA content of Kunlun 12 with PEG 6000 treatment. With increasing PEG 6000 concentration, the leaf RWC of Kunlun 12 gradually decreased (Figure 1B) and ABA content initially increased and then decreased (Figure 1C). The relationship between RWC and PEG 6000 concentration was linear, while the ABA content and PEG 6000 concentration relationship was a non-linear quadratic function. Therefore, it is possible to use PEG 6000 as an osmotic agent to simulate drought and water stress. The effects of PEG 6000 on leaf soluble protein content, relative conductivity, and malondialdehyde content were determined in Handizi, Kunlun 12, and Dama cultivars. The soluble protein content of the three cultivars initially increased and then decreased with increasing PEG 6000 concentration (Figure 2A). Compared with the control group, the protein content increased significantly at 5-15% PEG 6000 in Handizi and Kunlun 12, but decreased at 20-30% PEG 6000 in all three cultivars ($P<0.01$). However, in each group, the soluble protein content of Handizi was the highest, followed by Kunlun 12 and Dama. The results of relative conductivity and the malondialdehyde content were opposite those of the soluble protein content, with Handizi being the lowest, Kunlun 12 intermediate, and Dama the highest (Figure 2B and C). These results indicated that Handizi was the best drought-resistance cultivar, followed by Kunlun 12 and Dama.

Cloning and multiple sequence alignment of *HVA1s* from three cultivars

The cDNA sequences of the three *HVA1s* were 642 bp (Figure 3). Each of the cDNA sequences encoded 213 amino acids. Sequence comparison showed that the deduced amino acid sequence of *HVA1*-Handizi and *HVA1*-dama were identical, and *HVA1*-Kunlun12 was identical with them except for residue 197. Nine imperfect repeats of the 11 amino acids (Thr-Glu-Ala-Ala-Lys-Gln-Lys-Ala-Ala-Glu-Thr) were found in the three polypeptides.

Expression of the *HVA1* in hulless barley using semi-quantitative and quantitative polymerase chain reactions

The expression level of *HVA1* in the three varieties was very low in distilled water and 5% PEG 6000. However, the expression was considerably greater in 10~30% PEG 6000 (Figure 4A). The expression of the *HVA1* was detected by qPCR. The results showed that the expression of *HVA1* was not significantly different in the control and 5% PEG 6000 groups, which is consistent with the semi-quantitative PCR results. The expression of *HVA1* increased

Table 2. Sequences of primers used in this study.

Name	Sequence
P1	F:5 - TTTGGATCCATGGCCTCCAACCAGAACC -3
P2	R: 5 - GGGGAGCTCCGAACGACCAAACACGACT -3
P3	F: 5 - TCACGCTCAAGTACCCCATCGA -3
P4	R: 5 - GGAGCTGTTCTTGGCAGTCTCCA -3 F: 5 - GCAGCGTCCTCCAGCA -3 R: 5 - GGTGTTGTCCCCTCCCA -3 F: 5 - CAAGTATGTCATAGAGATTTGAA -3 R: 5 - GTAACCGAAGTCACAAATCT -3

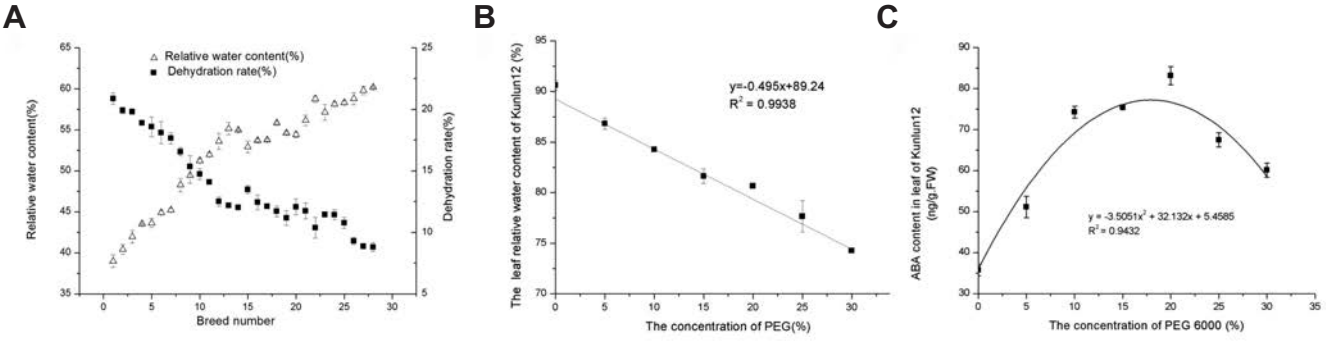

Figure 1. Resistance against water loss. A) Relative water content and dehydration rate of different varieties of hulless barley. The breed number indicates the different barley variety. B) The relationship between leaf relative water content of Kunlun 12 and polyethylene glycol (PEG) concentration. C) The relationship between leaf abscisic acid content of Kunlun 12 and PEG concentration. Error bars indicate the mean±standard deviation from three independent experiments.

from 10% to 30% in the PEG 6000 group, and reached the highest level in the 20-25% PEG 6000 treatment in three cultivars. The expression level of the *HVA1* in Handizi was the highest at each point, followed by Kunlun 12 and Dama, and the difference of expression level in the three cultivars was significant when treated with 10 to 30% solutions of PEG 6000. The highest expression level occurred at 25% PEG 6000 in Handizi and Kunlun 12, but at 20% PEG 6000 in Dama. Compared to the expression levels in 1% PEG, the highest transcription levels of *HVA1* were increased by 803-, 490- and 323-fold respectively (Figure 4B). The range of *HVA1* expression level in Handizi was wider than Kunlun 12 and Dama (Figure 4C). In addition, we tested the expression level of *HVA1* from 8 cultivars with 25% PEG 6000 treatment by qPCR. The expression level of *HVA1* increased from breed number 1 (Dama) to number 28 (Handizi) (Figure 4D). The difference of expression levels among the 8 cultivars was significant ($P<0.01$).

Discussion

Previous research demonstrated that a low PEG concentration promoted seed germination, seedling growth and improvement in the physiological function of hulless barley, while a high PEG concentration inhibited these functions (Yao and Wu, 2012). In this study we found that a low PEG concentration increased soluble protein content, but also decreased relative conductivity and malondialdehyde content. PEG 6000 is a non-ionic, water-soluble polymer, which does not rapidly penetrate intact plant tissues (Chazen *et al*., 1995). High MW PEG (6000-8000) is recommended for use in nutrient culture (Comeau *et al*., 2010; Blum, 2008). Therefore, it was reasonable to use PEG 6000 as an osmotic agent.

The functional roles and mechanisms of LEAs remain unclear. This drought-related candidate gene might be involved in plant adaptation to drought stress. It was identified through QTL analysis of Tadmor and ER/Apm Recombinant Inbreed Line (RIL) pop-

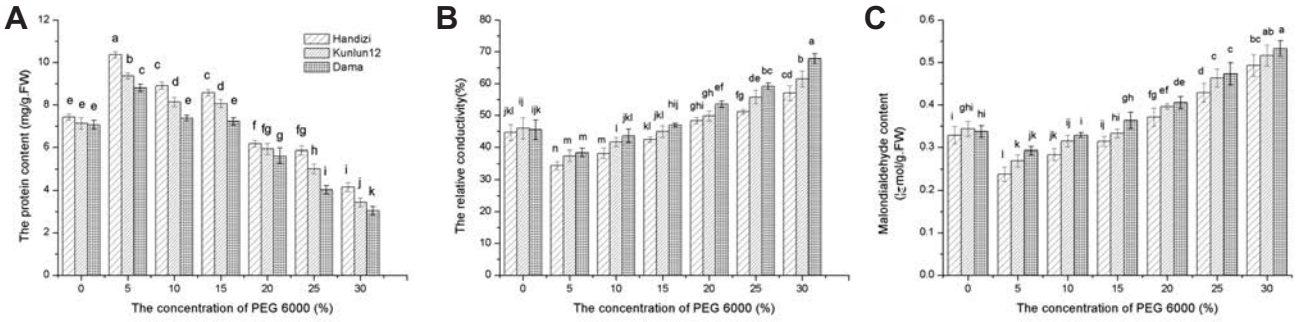

Figure 2. Drought resistance of Handizi, Kunlun12 and Dama. Effect of polyethylene glycol 6000 on leaf soluble protein content (A), relative conductivity (B) and malondialdehyde content (C) in Handizi, Kunlun 12 and Dama. Error bars indicate the mean±standard deviation from three independent experiments. Different letters indicate significant ($P<0.05$) differences between cultivars.

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HVA1-Handizi   MASNQNQGSYHAGETKARTEEKTGQMMGATKQKAGQTTEATKQKAGETAEATKQKTGETA 60
HVA1-Kunlun12  MASNQNQGSYHAGETKARTEEKTGQMMGATKQKAGQTTEATKQKAGETAEATKQKTGETA 60
HVA1-Dama      MASNQNQGSYHAGETKARTEEKTGQMMGATKQKAGQTTEATKQKAGETAEATKQKTGETA 60

HVA1-Handizi   EAAKQKAAEAKDKTAQTAQAAKDKTYETAQAAKERAAQGKDQTGSALGEKTEAAKQKAAE 120
HVA1-Kunlun12  EAAKQKAAEAKDKTAQTAQAAKDKTYETAQAAKERAAQGKDQTGSALGEKTEAAKQKAAE 120
HVA1-Dama      EAAKQKAAEAKDKTAQTAQAAKDKTYETAQAAKERAAQGKDQTGSALGEKTEAAKQKAAE 120

HVA1-Handizi   TTEAAKQKAAEATEAAKQKASDTAQYTKESAVAGKDKTGSVLQQAGETVVNAVVGAKDAV 180
HVA1-Kunlun12  TTEAAKQKAAEATEAAKQKASDTAQYTKESAVAGKDKTGSVLQQAGETVVNAVVGAKDAV 180
HVA1-Dama      TTEAAKQKAAEATEAAKQKASDTAQYTKESAVAGKDKTGSVLQQAGETVVNAVVGAKDAV 180

HVA1-Handizi   ANTLGMGGDNTSATKDATTGATVKDTTTTTRNH 213
HVA1-Kunlun12  ANTLGMGGDNTSATKDTTTGATVKDTTTTTRNH 213
HVA1-Dama      ANTLGMGGDNTSATKDATTGATVKDTTTTTRNH 213
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Figure 3. Multiple alignments of the HVA1 deduced amino acid sequences from Handizi, Kunlun 12 and Dama. The sequences were aligned using the ClustalW programme.

ulations (Du *et al*., 2004; Cseri *et al*., 2011). Under both salinity and drought stress, transgenic *HVA1* mulberry plants showed improved cellular membrane stability (CMS), higher photosynthetic yield, less photo-oxidative damage, and better water use efficiency compared to the non-transgenic plants (Lal *et al*., 2008). When the *HVA1* was transferred into maize, the transgenic plants had increased leaf relative water content (RWC), greater leaf and root biomass, and increased survival under complete 15 d drought while all wild-type non-transgenic control plants died (Nguyen and Sticklen, 2013). In malting barley genotypes, CO_2 assimilation rates and PSII efficiency in drought conditions were related to both water content and the accumulation of *HVA1* transcript in leaves (Rapacz *et al*., 2010). However, another group 3 LEA gene PcC3-06, isolated from *Craterostigma plantagineum*, failed to improve the drought resistance ability of transgenic tobacco (Hong *et al*., 1992). Accordingly, is there a link between *HVA1* and drought stress in hulless barley? What is the mechanism of *HVA1* in drought tolerance of hulless barley? Drought tolerance in barley was highly correlated with *HVA1* (Qian *et al*., 2007; Wójcik-Jagła *et al*., 2012). Our results also show this positive correlation. Variation in the drought resistance of hulless barley was caused by amino acid changes in *HVA1* (Qian *et al*., 2007). In this study, we selected three drought resistant cultivars from among 28 hulless barley cultivars according to their relative water content and dehydration rate. Then, we cloned *HVA1*s from the three cultivars, which were highly homologous at nucleotide and amino acid level with over 99% identities. We found that the expression level of the *HVA1* in drought-resistant cultivars was higher than expression in drought-sensitive hulless barley under the same water stress. Therefore, we suggest that different *HVA1* expression levels caused different levels of drought resistance in the three cultivars.

LEA protein is widely distributed in cells and plays an important role in stabilising cell membranes as a molecular barrier, combining ions, and protecting cells from oxidation. These functions are necessary for plant survival under high stress levels (Baker *et al*., 1988). LEA protein may also be a regulatory protein involved in plant osmotic adjustment and it may protect the endosperm and growing tissue from osmotic stress (Brini *et al*., 2007). Therefore, the protein produced by the *HVA1* is involved in osmotic regulation, possibly by protecting membranes from instability when the plant experiences water stress. The expression of the *HVA1* in hulless barley initially increased with an increase in osmotic stress,

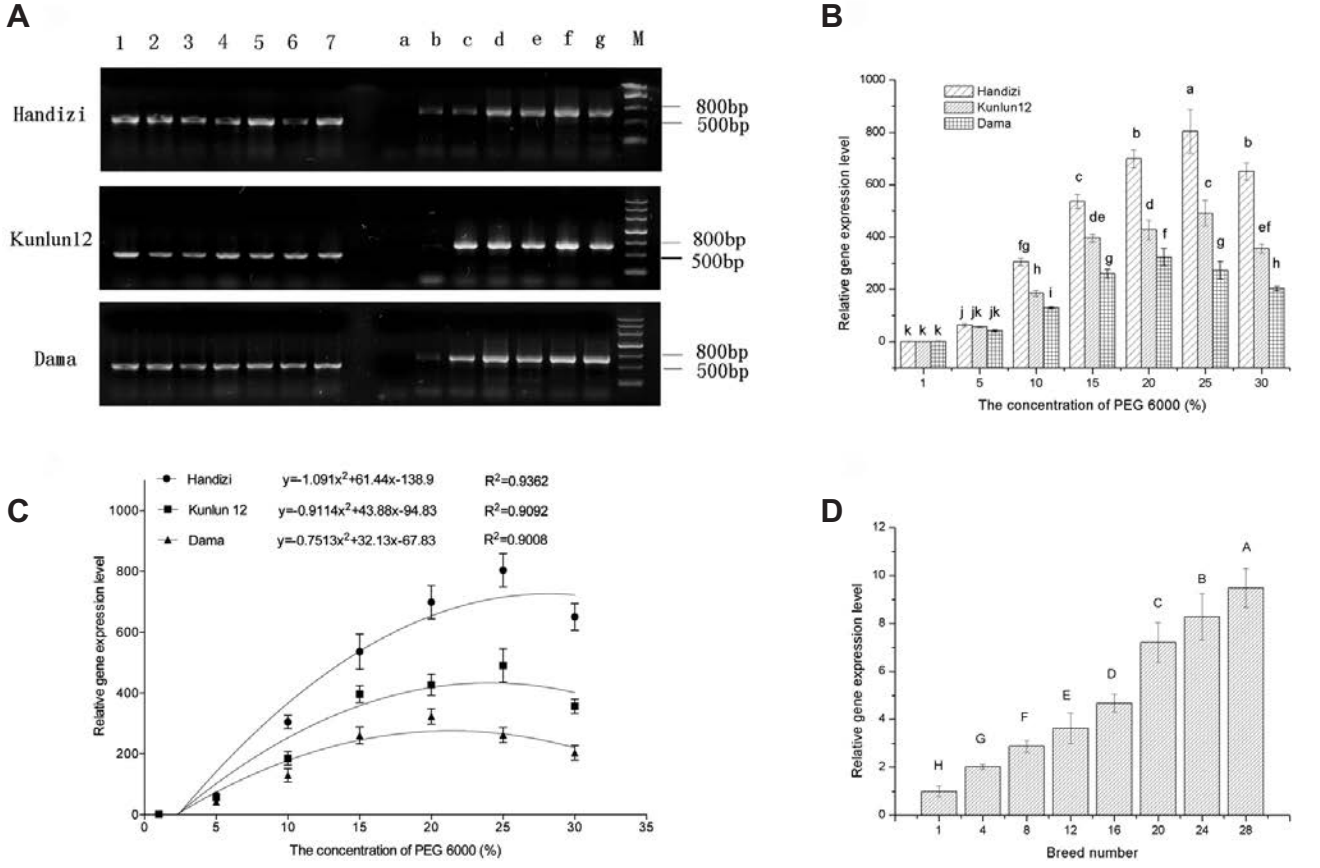

Figure 4. Expression levels of *HVA1* in hulless barley. Values for quantitative polymerase chain reaction are means±standard deviation of five replicates. Different letters indicate significant (P<0.05) differences between treatments. A) Relative expression levels of *HVA1* in Handizi, Kunlun 12 and Dama tested by RT-PCR. 1-7, Expression levels of β-Actin under PEG 6000 (0, 5, 10, 15, 20, 25 and 30%); a-g, expression levels of *HVA1* under PEG 6000 (0, 5, 10, 15, 20, 25 and 30%); M, marker. B) Relative expression levels of *HVA1* in Handizi, Kunlun 12 and Dama tested by quantitative real-time polymerase chain reaction (qRT-PCR). C) Expression profiling of *HVA1* under PEG 6000. D) Relative expression levels of *HVA1* in eight cultivars tested by qRT-PCR.

but decreased under prolonged stress. The reason might be that during the initial stages of the drought, plants need substantial LEA protein in order to rapidly stabilise and repair cytomembranes. Under continued water stress, the metabolic system of the plant may restrict the expression of the *HVA1*. This hypothesis was supported by qPCR results. Handizi had greater tolerance to PEG 6000 than Kunlun 12 and Dama. Compared to the expression levels in 1% PEG, the transcription levels of *HVA1* at its highest point increased by 803-, 490- and 323-fold in Handizi, Kunlun12, and Dama, respectively. However, the highest transcription levels in Handizi and Kunlun 12 were at 25% PEG 6000; in Dama highest transcription was at 20% PEG 6000. Also, based on regression formula extrapolation, *HVA1* was no longer expressed in 53.97% PEG 6000 in Handizi, 45.88% PEG 6000 in Kunlun 12, and 40.70% PEG 6000 in Dama.

Conclusions

Coping with the variability of biotic and abiotic stresses is essential in sustainable agriculture. Conventional breeding approaches can be used to develop improved varieties of hulless barley but the long time required supports the additional use of more precise biotechnological approaches. Genetic engineering techniques hold great promise for developing crop cultivars with drought tolerance (Checker *et al*., 2012). Understanding the mechanisms behind stress tolerance in crops under realistic conditions could accelerate drought resistance improvements in hulless barley. This study offers a process for identifying favorable cultivars and genetic controls of drought resistance in hulless barley. Drought resistance of plants is a quantitative character controlled by many genes, such as *OjERF* (Li *et al*., 2012) and *Dhn* (Saibi *et al*., 2015), but the *HVA1* appears to be a key component. Knowledge of the expression level of the *HVA1* under drought stress might be useful for breeding hulless barley with enhanced drought tolerance, but the interactions between *HVA1* and other drought resistance genes require further studies.

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