Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley HVA1 gene

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Abstract

The ABA-responsive barley gene HVA1, a member of group 3 late embryogenesis abundant (LEA) protein genes, was introduced into spring wheat (Triticum aestivum L.) cv. Hi-Line using the biolistic bombardment method. High levels of expression of the HVA1 gene, regulated by the maize abi1 promoter, were observed in leaves and roots of independent transgenic wheat plants and were inherited by offspring generations. T3 progenies of four selected transgenic wheat lines were tested under greenhouse conditions for tolerance of soil water deficit. Potted plants were grown under moderate water deficit and well-watered conditions, respectively. Two homozygous and one heterozygous transgenic lines expressing the HVA1 gene had significantly (P < 0.01) higher water use efficiency values, 0.66–0.68 g kg⁻¹, as compared to 0.57 and 0.53 g kg⁻¹, respectively, for the non-expressing transgenic and non-transgenic controls under moderate water deficit conditions. The two homozygous transgenic plant lines also had significantly greater total dry mass, root fresh and dry weights, and shoot dry weight compared to the two controls under soil water deficit conditions. Results of this study indicate that growth characteristics were improved in transgenic wheat plants constitutively expressing the barley HVA1 gene in response to soil water deficit. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Barley HVA1 gene; Biomass productivity; LEA protein; Water deficit; Water use efficiency; Wheat transformation

1. Introduction

Drought is an important environmental factor limiting the productivity of wheat and other crops world-wide. As irrigation water sources have become more scarce, development of crop cultivars with improved adaptation to drought is a major goal in many crop breeding programs. Of the physiological factors that affect drought tolerance in crops, the most important ones seem to be moderate water use through reduced leaf area and shorter growth duration, rooting ability to exploit deep soil moisture, the capacity for osmotic adjustment, and control of non-stomatal water loss from leaves [24]. Drought tolerance breeding might be facilitated by marker-assisted selection in the near future based on the development of molecular linkage maps for crop species [24].

When breeding for drought tolerance, biomass productivity and water use efficiency (WUE) are
considered important agronomic characters [1]. There is increasing interest in improving WUE of crop cultivars so that plants can grow and yield well under water deficient conditions [2,12]. In addition to classical breeding approaches, recent work has used genetic transformation to introduce various genes into plants for better tolerance to water deficit. One strategy is to genetically engineer plants to accumulate compatible osmolytes, such as amino acids, sugars, or sugar alcohols, resulting in decreased osmotic potential and avoidance of water deficit. Mannitol [29], proline [19], fructan [25], and trehalose [15] have been shown to accumulate in transgenic tobacco plants when genes encoding the corresponding biosynthetic enzymes were introduced, resulting in improved growth of transgenic plants under conditions of water deficit and/or salt stress. Additional novel roles of these compounds in the protection of plants against water stress were proposed [3,15].

Another transgenic approach is to constitutively over-express certain plant proteins, such as late embryogenic abundant (LEA) proteins that accumulate during seed desiccation, and in vegetative tissues when plants experience water deficit. Groups of LEA proteins have been defined based on their amino acid sequence homologies [10]. Three LEA proteins have been demonstrated to play roles in water deficit tolerance. LE25, a group 4 LEA protein from tomato (*Lycopersicum esculentum* L.) improved resistance to high salinity and freezing when expressed in yeast (*Saccharomyces cerevisiae*) [18], while *HVA1*, a group 3 LEA protein from barley (*Hordeum vulgare* L.) conferred tolerance to soil water deficit and salt stress in transgenic rice plants [34]. Very recently, a wheat LEA protein, Em, was demonstrated to function as an osmoprotective molecule in yeast [27].

The *HVA1* gene was first isolated from the aleurone layers of barley seeds as an ABA-inducible gene [16]. The deduced amino acid sequence from the corresponding cDNA indicates that *HVA1* encodes a 22 kDa protein which contains nine imperfect repeats of an 11 amino acid motif, a characteristic feature of group 3 LEA proteins. *HVA1* mRNA was rapidly induced in young seedlings (3 days after imbibition) by ABA and a series of stress conditions including partial dehydration, cold, NaCl, and heat. However, 7-day-old seedlings were much less responsive, indicating tight developmental control of *HVA1* gene expression [17]. Accumulation of *HVA1* protein in transgenic rice plants led to higher growth rates, delayed damage symptoms, and improved recovery (upon removal of the stress conditions) over non-transgenic controls when seedlings were subjected to water deficit and salt stress [34]. In tobacco, constitutive expression of the *HVA1* gene resulted in delayed wilting and wilting at lower leaf water contents (Ho, unpublished data).

In this study, we investigated the performance of transgenic wheat lines constitutively expressing the *HVA1* gene by measuring several agronomic characters under water deficient as well as under well-watered conditions. We found that three of four transgenic lines expressing the *HVA1* gene yielded more biomass and used water more efficiently than the control under moderate water deficient conditions. The engineered plants performed similarly to non-transgenic plants under well-watered conditions. Our results indicate that constitutive expression of *HVA1* gene helps improve wheat growth characteristics under water deficient conditions, and further demonstrate possible applications of certain LEA protein genes in engineering crop plants for tolerance to water deficit.

2. Materials and methods

2.1. Plant expression vectors

The plasmid expression vector pAB1 was constructed as described below (Fig. 1). A 1.1 kb

![Fig. 1. Plasmid map of transformation vector pAB1. The vector was based on plasmid pAHC17 [4] with barley *HVA1* gene driven by the maize *ubi1* promoter region (including the first exon and intron) and terminated by the *NOS* gene 3′ non-translated region. The *bar* gene was used as the selective marker. Abbreviations for the restriction sites: B: *Bam*HI, E: *Eco*RI, H: *Hind*III, N: *Nco*I.](image-url)
EcoRI fragment containing the full-length HVA1 cDNA [16] was filled using Klenow fragment and was blunt-end ligated to the previously digested and filled BanHI site of pAHC17 [4] to obtain pRQ42. A 2.09 kb HindIII fragment was obtained from pBARGUS [14] which contains the bar gene [6] driven by the CaMV 35S promoter, with the maize Adh1 intron 1 in the 5′ non-translated region and the NOS terminator. This fragment was ligated with HindIII-digested pRQ42 to obtain pAB1. The bar gene encodes the enzyme phosphinothricin acetyl transferase (PAT) which inactivates phosphinothricin, the active ingredient of the herbicide bialaphos [6].

2.2. Wheat transformation

Immature embryos were isolated from greenhouse-grown wheat (Triticum aestivum L.) cv. Hi-Line, an elite Montana spring wheat variety [21] and precultured for 1–4 days in the dark on modified MS medium as reported by Weeks et al. [32] before bombardment. Preparation of gold particles and coating with plasmid DNA was carried out based on the manufacturer’s instructions (Bio-Rad, Hercules, CA, USA). Osmotic treatment of target tissue before and after bombardment was performed according to Vain et al. [30]. Bombarded tissue was placed on the same culture medium supplemented with 5 mg l⁻¹ bialaphos (a gift from Dr H. Anzai, Meiji Seika Kaisha, Japan) for 4 weeks at 25°C in the dark. Bialaphos-resistant calli were transferred to regeneration medium (MS medium containing 2% sucrose, 0.15 mg l⁻¹ thidiazuron and 1 mg l⁻¹ bialaphos) for 2–3 weeks at 25°C under a 16 h photoperiod (66 μmol m⁻² s⁻¹). After ≈2 weeks, regenerated shoots were transferred to Magenta® boxes (Sigma, St Louis, MO, USA) containing rooting medium (half-strength MS medium and 2 mg l⁻¹ bialaphos) for 2–4 weeks at 25°C under the above light conditions.

Plantlets were transferred from rooting medium to greenhouse potting mix (Sunshine mix number 1; Fison’s, Canada) and were covered with beakers for the first few days after transplantation to prevent desiccation. Greenhouse day/night temperatures were 25 ± 2/19°C under a 16 h photoperiod with supplemental lights to provide 150 μmol m⁻² s⁻¹ light intensity. Herbicide resistance of primary transformants and progeny was tested by a leaf painting assay and/or spraying with a 1000-fold dilution of the commercial herbicide Glufosinate 200™ (AgrEvo, NJ, USA) containing 20% ammonium glufosinate.

2.3. Southern blot hybridization

Genomic DNA was extracted from plant tissue as described by Dellaporta et al. [7] with minor modifications. Thirty micrograms of genomic DNA from each plant examined was digested with NcoI restriction enzyme, electrophoresed in 1% agarose gels and transferred to a Hybond N+ nylon membrane (Amersham, Arlington Heights, IL, USA). Further processing, prehybridization, hybridization and washes of the blots were performed according to the manufacturer’s recommendations. The two final washes were made in 0.1 × SSPE and 0.1% SDS at 65°C for 15 min. Due to the existence of homologous HVA1 gene(s) in wheat, the blotted DNA was probed with the [α-32P] dCTP labeled maize ubi1 promoter plus the first exon and intron sequences cleaved from pAHC17 with BanHI and HindIII to confirm transgene insertion. Probes were produced using a Prime It II random priming kit (Stratagene, La Jolla, CA, USA) to a specific activity of >1 × 10⁹ dpm μg⁻¹.

2.4. Protein analysis of transgenic plants

Expression of HVA1 protein was determined during development of the transgenic plants. Embryo tissue samples were obtained 18 h, 2 and 3 days after imbibition while leaf tissue samples were collected 5, 21 and 40 days after imbibition. Approximately 100 mg of the plant tissue was ground to a paste and suspended in 100 μl of protein extraction buffer (0.125 M Tris–HCl, pH 6.8, 2.5% SDS, 10% glycerol) in a microfuge tube. Extracts were incubated at 100°C for 5 min, centrifuged at 13 000 × g for 5 min at room temperature and supernatants collected. Total protein content was estimated using a BCA protein assay kit (Pierce, IL, USA) with BSA as a standard. All protein samples were flash frozen in liquid nitrogen and stored at −20°C until western analysis. Twenty-five to thirty micrograms of total protein was loaded per lane for 15% SDS-PAGE. Western blotting was performed using the BIO-RAD Mini Trans-Blot apparatus according to the manufac-
turer's instructions. Processing of the western blots was performed following instructions in the Promega Protocols and Applications Guide (1991). Nitrocellulose membranes were incubated in anti-\textit{HVA1} antibody [17] at 1:25 000 dilution for 30 min followed by incubation in goat anti-rabbit IgG alkaline phosphatase conjugate (Promega, Madison, WI, USA). The \textit{HVA1} protein was detected using 4-nitroblue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrates (Promega).

2.5. Water deficit and well-watered treatments of transgenic plants

T₃ seeds of transgenic wheat lines were used for water deficit experiments. Two seeds of each line, as well as controls, were planted in a plastic pot of 5130 cm³ containing ≈ 4000 g of dry soil in the greenhouse. Eight replicated pots were used for each transgenic line and the control. The pots were well watered every other day until the seedlings emerged. Fifteen days after germination, the less vigorous seedling was removed from each pot leaving one plant per pot. A controlled watering program was started at this point. Soil used for the experiments was a 1:1 mixture of washed concrete sand and Bozeman silt loam mixed with peat moss (1:1, v/v) with a final organic matter content of 30%. Soil water retention measurements were obtained using a laboratory pressure plate apparatus [20]. The measured data were then fitted to van Genuchten's [31] parametric model. Mass water content (kg/kg) corresponding to soil matric potentials of −20 and −500 kPa were determined from the retention curve to simulate well-watered and moderate water deficit treatments, respectively. It was experimentally determined that addition of 500 and 100 ml of water every other day was required to maintain the desired soil matric potentials for plants up to 8–10 weeks. Soil water status thus presumably became progressively slightly ‘drier’ (lower matric potential) than the target levels as plants grew further and used more soil water. These water volumes were manually added to each pot 38 times during the experiment. Total water usage of each pot was calculated as: amount of water added each time multiplied by number of watering times (500 ml × 38 or 100 ml × 38, respectively). All plants received fertilizer (Peters Professional 20:20:20 GP by Scotts, OH, USA) to deliver 200 ppm N at weekly intervals.

2.6. Analysis of growth and biomass

Plants were harvested when the primary stem turned yellow. Shoots were removed at the soil surface and roots were carefully washed and blotted dry before measuring fresh weight. Dry weight was obtained after drying plant tissue at 65°C for 7 days.

2.7. Data collection and statistical analysis

Water use efficiency (WUE) means were calculated as the ratios of the total dry mass (TDW) produced over the total water used (TWU) [2,13]. For segregating lines (lines 344 and 357, as indicated by western blotting of \textit{HVA1} expression), only plants that expressed the \textit{HVA1} protein (7 plants for line 344 and 6 for line 357) were included in data analysis. Analysis of variance was performed using TurboStat version 1.12, developed by the Department of Agronomy, Oklahoma State University, with treatment means separated by LSD (\(P = 0.05\) or 0.01).

3. Results

3.1. Molecular analyses of transgenic wheat

A total of ten independent transgenic T₀ plants were generated from transformation experiments, as indicated by leaf painting assays and PCR analyses for presence of the \textit{bar} gene. Of the ten primary transgenic plant lines, five (lines 1, 84, 111, 344 and 357) were selected for further characterization based on normal plant morphology and fertility in subsequent generations. Among the T₃ generation lines tested, lines 1 and 111 were homozygous as indicated by uniform resistance in leaf painting assays in the previous generation as well as the \textit{HVA1} protein expression in this generation (data not shown). Lines 344 and 357 were still segregating as indicated by \textit{HVA1} protein expression. Line 84 did not express \textit{HVA1} protein and was used as a non-expressing, transgenic control while plants of parental cv. Hi-Line were used as non-transgenic controls. Because the wheat genome contains homologues of the barley \textit{HVA1} gene [5], a probe that contained promoter, first exon, and first intron of the maize \textit{ubi1} gene was used to detect the presence of
the transgene in Southern blot analyses. Hybridization was observed in high molecular weight, non-digested genomic DNA of the transgenic plants, indicating that the transgene was integrated into the chromosomal DNA (data not shown). The restriction enzyme NcoI has two cleavage sites on the pAB1 plasmid: one within the HVA1 coding sequence and the other within the ubi1 promoter to liberate a 1.85 kb fragment (Fig. 1). When genomic DNA was digested with NcoI, the 1.85 kb fragment was observed in all transgenic lines except line 84 (indicating that line 84 did not contain the intact copy of HVA1 transgene construct, Fig. 2). Other hybridizing fragments of varying sizes were observed suggesting specific transgene integration patterns in each line and confirming that the transgenic lines resulted from independent transformation events.

3.2. Accumulation of HVA1 protein in transgenic wheat

Detection of transgenic HVA1 protein with the HVA1 antibody during the first three days of imbibition was confounded by the presence of wheat HVA1 homologues [5] that cross-reacted with the antibody. Five major protein bands (≈ 27–19 kDa) reacted with the HVA1 polyclonal antibody in protein extracts from wheat (Fig. 3(a, b, c)) while three major bands (22, 19.4, and 18.4 kDa, respectively) were detected in control barley samples for the same time period. In non-transgenic wheat and in transgenic line 84, expression of wheat HVA1 homologues was apparent up to 72 h after seed imbibition. However, there were no
such proteins detected as the plants developed further. In transgenic line 1 (Fig. 3(c)), \( \approx 22 \text{kDa} \) and two smaller bands (\( < 19 \text{kDa} \)) were observed in extracts from 5, 21 and 40 day old plants while no such bands were detected in control plants of the same age, indicating that these proteins represent the barley \( HVA1 \) transgene products. The same expression pattern was observed in other transgenic plant lines (including lines 111, 344, 357) and their progenies (data not shown). Thus, these plants were considered expressors of the \( HVA1 \) transgene.

3.3. Growth performance and WUE of transgenic wheat plants

Soil matric potentials of \(-20\) and \(-500 \text{kPa}\) were designed to simulate well-watered and moderate water deficit treatments, respectively. All plants under water deficit treatment showed poor growth as evidenced by the lower number of tillers (averaged 2.6–3.4) in each plant line compared to that of the well watered plants (averaged 7.2–12), and the height of the plants (about 10–15\% shorter than the well watered plants). In moisture stressed plants, seed yield came mainly from the primary stem. The leaves in general had a dry and leathery appearance. No color difference between the leaves of plants under water deficit treatment and those of well watered plants was observed. Roots of plants under water deficit treatment were also distinctly shorter with less fibrous branching in all lines. In an attempt to measure the rate of root growth in selected transgenic lines under water deficit treatment, plants were grown in soil in special glass lined wooden pots tilted at a 45\(^\circ\) angle. The rate at which the roots grew was monitored for about 6 weeks following seed germination. No significant difference was observed between transgenic lines and controls. Overall, not much morphological difference was observed between transgenic and control plants under each treatment.

However, significant differences in agronomic traits were observed between transgenic and control plants receiving water deficit treatment when measurements were performed after harvest. Of the four \( HVA1 \)-expressing transgenic lines tested (Table 1(a)), homozygous lines 1 and 111 and heterozygous line 344 showed substantial increases in root fresh weight (RFW, by 18–55\%), root dry weight (RDW, by 57–60\% for lines 1 and 111), shoot dry weight (SDW, by 13–17\%), total dry mass (TDM, by 16–19\%) and seed weight (SW, in line 111 only) over the non-transgenic and the non-expressing transgenic (line 84) controls under moderate water deficit conditions. Among these, the increases in RFW, RDW, and TDM of the homozygous lines 1 and 111 were statistically significant (\( P < 0.01 \)) compared to those from the non-transgenic control plants. Furthermore, the water use efficiency (WUE) of lines 1, 111 and 344 (0.679, 0.675 and 0.662 g kg\(^{-1}\), respectively) was significantly improved (\( P < 0.01 \)) over the controls (0.527 and 0.568 g kg\(^{-1}\), respectively). Line 357 performed similarly to control plants. All transgenic offspring plants tested had normal phenotypes as compared to the parental control plants.

Under well-watered growing conditions (Table 1(b)), transgenic and non-transgenic plants performed similarly overall except the non-expressing, transgenic control line 84 which grew poorly for unknown reasons. Shoot dry weight of line 1 was significantly (\( P < 0.05 \)) better than the non-transgenic. Lines 111 and 344 had decreased root dry weight and seed weight compared to the controls.

4. Discussion

In this study, we characterized transgenic wheat lines expressing a barley \( HVA1 \) gene for their performance under soil water deficit and well-watered conditions. Three out of the four lines tested displayed improvement in important agronomic traits, including total dry mass and water use efficiency, shoot dry weight, root fresh and dry weights when the plants were grown under soil water deficit conditions. Although no difference was detected in root growth rate, the root dry weight increases in lines 1 and 111 are remarkable and may have played an important role in the observed tolerance to soil water deficit. It is particularly interesting to note the significant improvement in biomass productivity and WUE in these three lines (see discussion below). Since our experiments were primarily designed to obtain the biomass and water use efficiency data, plants were harvested before the grain maturation. Thus, seed weight obtained in this study may not reflect actual yield and therefore should be considered cautiously.
Table 1
Growth performance of transgenic wheat plants exposed to moderate water deficit or well-watered conditions in the greenhouse\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Line</th>
<th>Tillers (number)</th>
<th>RFW (g)</th>
<th>RDW (g)</th>
<th>SDW (g)</th>
<th>TDM (g)</th>
<th>SW (g)</th>
<th>WUE (\text{g kg}^{-1})</th>
<th>TDM, TWU (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{(a) Treatment under moderate water deficit conditions}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{c}</td>
<td>3.4</td>
<td>1.454**</td>
<td>0.396**</td>
<td>2.183**</td>
<td>2.581**</td>
<td>0.750</td>
<td>0.679**</td>
<td></td>
</tr>
<tr>
<td>111\textsuperscript{c}</td>
<td>2.8</td>
<td>1.655**</td>
<td>0.406**</td>
<td>2.160**</td>
<td>2.566**</td>
<td>0.867*</td>
<td>0.675**</td>
<td></td>
</tr>
<tr>
<td>344\textsuperscript{d}</td>
<td>2.8</td>
<td>1.325</td>
<td>0.286</td>
<td>2.227**</td>
<td>2.515**</td>
<td>0.728</td>
<td>0.662**</td>
<td></td>
</tr>
<tr>
<td>357\textsuperscript{d}</td>
<td>3.0</td>
<td>1.115</td>
<td>0.263</td>
<td>1.800</td>
<td>2.083</td>
<td>0.667</td>
<td>0.548</td>
<td></td>
</tr>
<tr>
<td>84 (CK1)\textsuperscript{e}</td>
<td>2.6</td>
<td>1.278</td>
<td>0.244</td>
<td>1.757</td>
<td>2.001</td>
<td>0.681</td>
<td>0.527</td>
<td></td>
</tr>
<tr>
<td>Hi-Line (CK2)\textsuperscript{f}</td>
<td>3.2</td>
<td>1.103</td>
<td>0.253</td>
<td>1.907</td>
<td>2.160</td>
<td>0.707</td>
<td>0.568</td>
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</tr>
<tr>
<td>LSD 5%</td>
<td>1.2</td>
<td>0.240</td>
<td>0.081</td>
<td>0.219</td>
<td>0.245</td>
<td>0.139</td>
<td>0.064</td>
<td></td>
</tr>
<tr>
<td>LSD 1%</td>
<td>1.6</td>
<td>0.320</td>
<td>0.110</td>
<td>0.297</td>
<td>0.332</td>
<td>0.189</td>
<td>0.087</td>
<td></td>
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<tr>
<td>\textit{(b) Treatment under well watered conditions}</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{c}</td>
<td>12.0**</td>
<td>6.669**</td>
<td>2.154**</td>
<td>13.463**</td>
<td>15.617**</td>
<td>4.394**</td>
<td>0.822**</td>
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<tr>
<td>111\textsuperscript{c}</td>
<td>11.0**</td>
<td>6.408**</td>
<td>1.475</td>
<td>11.670**</td>
<td>13.145**</td>
<td>2.493</td>
<td>0.692**</td>
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</tr>
<tr>
<td>344\textsuperscript{d}</td>
<td>7.2</td>
<td>5.850**</td>
<td>1.410</td>
<td>11.006**</td>
<td>12.416**</td>
<td>2.292</td>
<td>0.653**</td>
<td></td>
</tr>
<tr>
<td>357\textsuperscript{d}</td>
<td>9.5**</td>
<td>7.503**</td>
<td>1.987**</td>
<td>11.167**</td>
<td>13.153**</td>
<td>3.613**</td>
<td>0.692**</td>
<td></td>
</tr>
<tr>
<td>84 (CK1)\textsuperscript{e}</td>
<td>7.4</td>
<td>3.959</td>
<td>1.067</td>
<td>8.344</td>
<td>9.411</td>
<td>2.485</td>
<td>0.495</td>
<td></td>
</tr>
<tr>
<td>Hi-line (CK2)\textsuperscript{f}</td>
<td>11**</td>
<td>6.092**</td>
<td>2.100**</td>
<td>11.954**</td>
<td>14.054**</td>
<td>3.964**</td>
<td>0.740**</td>
<td></td>
</tr>
<tr>
<td>LSD 5%</td>
<td>1.5</td>
<td>1.310</td>
<td>0.470</td>
<td>1.348</td>
<td>1.612</td>
<td>0.715</td>
<td>0.085</td>
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<tr>
<td>LSD 1%</td>
<td>2.1</td>
<td>1.775</td>
<td>0.637</td>
<td>1.826</td>
<td>2.184</td>
<td>0.968</td>
<td>0.115</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} The values are means from eight replicates in each line.
\textsuperscript{b} RFW: root fresh weight; RDW: root dry weight; SDW: shoot dry weight; TDM: total dry mass; SW: seed weight; WUE: water use efficiency; TWU: total water used.
\textsuperscript{c} Transgenic lines 1 and 111 are homozygous.
\textsuperscript{d} Transgenic lines 344 and 357 are heterozygous.
\textsuperscript{e} Line 84 (CK1): non-expressing, transgenic line as a control.
\textsuperscript{f} Hi-Line (CK2): non-transgenic control.
\textsuperscript{†} Significant difference over CK1 at \(P = 0.05\) level.
\textsuperscript{††} Significant difference over CK1 at \(P = 0.01\) level.
* Significant difference over CK2 at \(P = 0.05\) level.
** Significant difference over CK2 at \(P = 0.01\) level.

Biomass (total dry matter) production is an important criterion to judge drought tolerance in crop breeding [23] while WUE is the total dry matter produced by the plants per unit of water used. The higher the production per unit of water used, the higher the efficiency [2]. The fact that three out of four transgenic wheat lines had significantly higher \((P < 0.01)\) total dry matter and water use efficiency strongly indicates that these transgenic lines are more tolerant of soil water deficit than the control plants. The observed difference was attributed to the \textit{HVA}1 transgene. More investigation is needed to reveal what physiological processes have been altered in these transgenic plants to improve the biomass productivity and the water use efficiency.

It is notable in our experiments that homozygous lines seemed to display more tolerance to soil water deficit than the heterozygous lines in general. No significant difference in \textit{HVA}1 protein level can be detected among these lines in western blot analysis. Similar observations were noted with \textit{HVA}1 transformed tobacco plants where the tolerance effect was only evident when the \textit{HVA}1 transgene was homozygous (Ho, unpublished data). Thus, the effect of \textit{HVA}1 transgene homozygosity remains to be elucidated.

The \textit{HVA}1 protein molecular weight is disputed in different reports. Hong et al. [17] reported a 27 kDa peptide from in vitro transcription and translation of the cDNA clone although the reading frame actually encodes a 22 kDa polypeptide. Xu et al. [34], who used the same cDNA, demonstrated a 27 kDa protein in transgenic rice. In this work, we detected a major 22 kDa protein and two other minor proteins less than 19 kDa in transgenic wheat plants. \textit{HVA}1 proteins with a similar molecular weight were observed with the
control barley sample, indicating the authenticity of transgene expression in wheat. Possible reasons for these discrepancies could be: (1) we used a different extraction method which may help the protein migrate in electrophoresis to the position that reflects its original molecular weight; or (2) the HVA1 protein was less stable and was degraded during extraction and storage as shown in our HVA1 transgenic wheat plants (data not shown).

Our experiments provide further evidence to support the role of group 3 LEA proteins in water stress protection. The group 3 LEA proteins are composed of tandem repeats of an 11 amino acid motif that may form an amphiphilic alpha helix structure. Although a series of group 3 LEA protein genes have been isolated and their accumulation has been correlated with water stress [8,9,11,22,26,28,33], the hypothesized protective role has been confirmed, at least for HVA1, by the transgenic work by Xu et al. [34] and our investigations. However, the exact function of group 3 LEA proteins is still not clear although many hypotheses have been put forth [3]. Further investigation of the physiological processes in our transgenic wheat plants may provide clues to the mechanism(s) involved as well as to the potential function of the HVA1 protein. Meanwhile, more biochemical studies of the group 3 LEA proteins are needed to establish a structure-function relationship of this group of important proteins as well as the mechanism of their protective roles. Considering the strict developmental regulation of these genes, transgenic plants and cells that constitutively overexpress LEA3 genes, such as the HVA1 gene, may offer a favorable system for such investigations.

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