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Competence of oat (*Avena sativa* L.) shoot apical meristems for integrative transformation, inherited expression, and osmotic tolerance of transgenic lines containing *hva1*

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Abstract Three oat (*Avena sativa* L.) cultivars have been successfully transformed using an efficient and reproducible in vitro culture system for differentiation of multiple shoots from shoot apical meristems. The transformation was performed using microprojectile bombardment with two plasmids (pBY520 and pAct1-D) containing linked (*hva1-bar*) and non-linked (*gus*) genes. The *hva1* and *bar* genes cointegrated with a frequency of 100% as expected, and 61.6% of the transgenic plants carried all three genes. Molecular and biochemical analyses in R0, R1 and R2 progenies confirmed stable integration and expression of all transgenes. Localization of the GUS protein in R0 and R1 plants revealed that high-expression of *gus* occurred in vascular tissues and in the pollen grains of mature flowers. The constitutive expression of HVA1 protein was observed at all developmental stages of transgenic plants, and was particularly stronger during the early seedling stages. R2 progeny of five independent transgenic lines was tested in vitro for tolerance to osmotic (salt and mannitol) stresses. As compared to non-transgenic control plants, transgenic plants maintained a higher growth and showed significantly ($P < 0.05$) increased tolerance to stress conditions. Less than 10% of transgenic plants showed symptoms of wilting or death of leaves and, when these symptoms present were delayed in transgenic plants as compared to 80% of non-transgenic plants, either wilted or died. These symptoms confirmed the increased in vitro tolerance in *hva1*-expressing transgenic plants to non-transgenic plants, providing strong evidence that the HVA1 protein may play an important role in the protection of oats against salinity and possible water-deficiency stress conditions.

Keywords Oat (*Avena sativa* L.) · *hva1* · *bar* · *gus* · Shoot apical meristem · Salt- and mannitol-stress

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Introduction

Increased soil-salinity and water-deficiency are major environmental stresses limiting plant growth and hampering agriculture worldwide (Epstein et al. 1980; Yancey et al. 1982). To circumvent environmental stresses, plants have developed different physiological and biochemical mechanisms. In response to osmotic stresses, plants produce low-molecular-weight metabolic compounds such as sugar alcohols and special amino acids as a mechanism to adapt to, or tolerate, stress conditions (Greenway and Munns 1980; Yancey et al. 1982). In addition to these metabolic changes, it has also been observed that, under stress conditions, certain genes are induced and some new proteins are synthesized in plants (Skriver and Mundy 1990; Thomashow and Browse 1999). The adaptation mechanism to stress tolerance varies with different plant species or even within the same species (Verma and Yadava 1986).

Oat (*Avena sativa* L.) is a temperate cereal crop and is a component of human and animal diets (Forsberg and Shands 1989). Oat is widely adapted to different soil types and climatic conditions, and it requires sufficient water for growth and grain production. The yield of oat grain increases linearly with the availability of sufficient water and it decreases as the soil water-deficiency is experienced during the growth season (Martin et al. 2001). Oat is also sensitive to hot dry weather. Information on

the salt tolerance of oat cultivars is limited. Soil salinity adversely affects seed germination and subsequent development in different oat cultivars (Murty et al. 1984; Verma and Yadava 1986). Oat is considered to be a moderately salt-tolerant species compared with other cereal or forage crops (Murty et al. 1984). Wise selection of varieties with genetic potential to produce satisfactory yields under stress conditions may help to improve oat production.

HVA1, is a group-3 Late Embryogenesis Abundant (LEA) stress-related protein from barley (*Hordeum vulgare* L.), which is specifically expressed in the aleurone layers and the embryos during late seed development (Hong et al. 1988). Expression of the *hva1* gene is rapidly induced in young seedlings under several stress conditions, such as dehydration, salt and extreme temperatures or by abscisic acid (ABA) treatment (Hong et al. 1992). The function of HVA1 protein in stress protection has been investigated using a transgenic approach in rice (Xu et al. 1996) and wheat (Sivamani et al. 2000).

In the last decade, major achievements in the improvement of cereal crops have been made via the transformation of useful trait genes (Mazur et al. 1999). However, very few reports are available that describe the genotype independency for in vitro regeneration and genetic engineering systems of oat. Previously, oat was transformed with the Biolistc mediated-transformation system using mature embryo-derived callus as a target tissue (Torbert et al. 1998). In that approach, the selectable marker neomycin phosphotransferase II (*nptII*) gene was used to study transformation efficiency. In another similar approach, transformation of mature oat embryo-derived callus was performed using hygromycin phosphotransferase (*hpt*) and β-glucuronidase (*gus*) genes (Cho et al. 1999). However, the application of the embryo-derived callus system to routine oat transformation may not be feasible due to the limited number of genotypes that are capable of transformation, and the undesirable somaclonal variations caused by the prolonged tissue culture (Somers 1999).

A system to produce multiple shoot primordia from cereal shoot apical meristems was developed in our laboratory (Zhong et al. 1992, 1998; Zhang et al. 1996; Devi et al. 2000; Ahmad et al. 2002). The efficient recovery of transgenic maize plants via this system (Zhong et al. 1996) suggested that this apical meristem system could be extended to develop a highly efficient and reproducible system for oat transformation (Zhang et al. 1996). This system may have certain advantages, such as the use of mature dry seeds to produce multiple shoot meristem cultures, the high regeneration ability of shoot apical meristems, and the high level of fertility and genomic stability in regenerated transgenic plants. These are advantages over the system previously employed for oat transformation that utilizes the bombardment of mature embryo-derived oat callus, as was described by Zhang et al. (1999).

Here, we describe the competence of multiple shoot cultures of three different oat cultivars derived from shoot apical meristems for Biolistic-mediated genetic transformation with the barley *hva1* gene. We also studied the osmotic-stress (salt and mannitol) tolerance of transgenic oat cultivars constitutively expressing HVA1. Finally, we present data on the regenerability, fertility, and stability of transgene expression in transgenic oat progenies.

Materials and methods

Plant materials

Following our previous work on the shoot multiplication of different oat cultivars (Zhang et al. 1996), three oat cultivars, Ogle, Pacer and Prairie, were used for transformation. Seeds were surface-sterilized with 70% ethanol followed by 20% clorox bleach (Clorox professional Products Company, Oakland, Calif., USA) and germinated on MS (Murashige and Skoog 1962) basal medium (Sigma-Aldrich) as described by Zhang et al. (1996). Shoot apices, containing apical meristems, two to three leaf primordia, and 3–5-mm-length leaf bases, were isolated from 1-week-old seedlings and cultured on shoot multiplication medium [MS basal medium supplemented with 500 mg/l of casein enzymatic hydrolysate, 3% sucrose, 0.5 mg/l of 2, 4-D (2,4-Dichlorophenoxyacetic acid), 2 mg/l of BA (N6-Benzyladenine) and 0.3% phytagel, pH 5.6].

Plasmids

Two plasmids, BY520 (Xu et al. 1996) and Act1-D (Zhang et al. 1991), were used. The plasmid BY520 contained a selectable marker/herbicide resistant *Steptomyces hygroscopicus bar* (Phosphinothricin acetyl transferase) gene and the barley *hva1* gene (Fig. 1). The selectable marker *bar* is driven by CaMV35S (cauliflower mosaic virus promoter) and the nos (*Agrobacterium* nopaline synthase gene) 3′ non-coding region, while *hva1* is driven by Act1 (5′ region of the rice actin 1 gene) and terminated with the pin II (Potato protease inhibitor II gene) 3′ non-coding region. The plasmid Act1-D contained the *Escherichia coli* β-glucuronidase (*gus*) gene flanked by the Act1 promoter and the nos terminator (Fig. 1).

In vitro multiple shoot transformation

One-month-old multiple shoot cultures, differentiated from the shoot meristems of multiple shoots, were used for transformation experiments. Multiple shoot cultures were physically exposed by removal of the coleoptiles and leaves, before bombardment if necessary. Plasmid DNA (15 μg of pBY520 and pAct1-D at a 1:1 molar ratio) was precipitated onto tungsten particles as described (Zhong et al. 1996). Bombardment was carried out using a Biolistic Gun (PDS 1,000/He, Bio-Rad) at 1,550 psi.

Bombarded multiple shoot cultures were transferred to shoot multiplication medium without selection for 4 weeks in continuous light with one subculture. The shoot clumps were then divided and transferred to shoot multiplication medium containing 5 mg/l of glufosinate ammonium to select the transformants. After 4 weeks, with one subculture on selection, green shoot clumps were further divided and subcultured on selection medium containing 10 mg/l of glufosinate ammonium for 4–6 weeks. After a total of 4 months of selection and multiplication medium, fast-growing multiple shoot clumps were transferred to shoot-elongation medium (MS basal medium, 2% sucrose and 0.3% Phytagel, pH 5.6) containing 15 mg/l of glufosinate ammonium, for vegetative and root development. Regenerated putative transgenic plants at 5–10 cm length with 2–3 leaves were transferred to soil and grown in a greenhouse until maturity. Transgenic plants were self-polli-

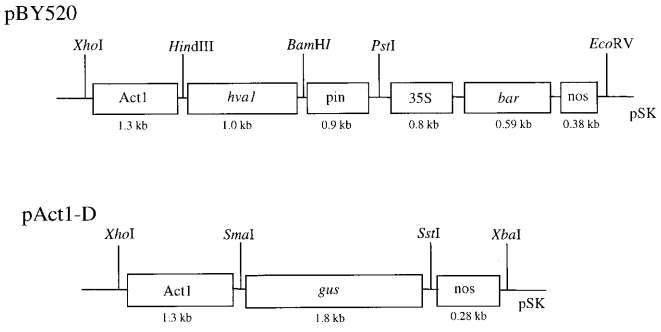

Fig. 1 Schematic representation of the plasmids. *Act1*: rice actin promoter; *hva1*: *hva1* gene from barley; *pin*: pin transcription terminator; *35S*: CaMV 35S promoter; *bar*: coding sequence of the *bar* gene; *nos*: nos transcription terminator; *gus*: coding sequence of the GUS gene

nated and seeds from each plant were harvested and stored separately at 4 °C.

Production and analysis of progeny

Screening of seeds generated by the self-polination of transgenic R0, R1 and R2 progenies containing *bar* was performed either through germination on half-strength MS basal medium containing 15 mg/l of glufosinate ammonium or by spraying 1% Ignite herbicide on greenhouse-germinated seedlings. The segregation data were statistically analyzed by a χ^2 test (Strickberger 1985).

Histochemical analysis of GUS

Different plant tissues and organs from the transgenic and non-transgenic plants were used to detect GUS expression. Samples were immersed in GUS substrate mixture and incubated at 37°C as described elsewhere (Jafferson et al. 1987). Tissues were prepared using free-hand cross sections and were examined under a Zeiss SV8 stereomicroscope according to Zhong et al. (1996).

Molecular analysis of transgenic plants

Confirmation of transgene integration into oat plant genomes was performed by Southern-blot hybridization (Southern 1975) using the *hva1*-coding sequence as a probe. Genomic DNA from transgenic and non-transgenic oat plants was isolated using the Phytopure Plant DNA extraction kit (Amersham-Pharmacia Biotech). For Southern blots, 10 μg of genomic DNA from R0, R1 and R2 transgenic oat plants was digested with *Hin*dIII or *Hin*dIII-*Bam*HI restriction enzymes and fractionated on a 0.8% agarose gel. Gels were denatured, neutralized, and blotted onto Hybond-N^+ membranes (Amersham-Pharmacia Biotech). A gene-specific probe was generated using a *Hin*dIII-*Bam*HI digest of pBY520 to isolate a 1.0-kb fragment containing the *hva1*-coding sequence. The restriction fragment was purified using the QIAquick kit (QIAGEN), and labeled with α-[^{32}P]-dCTP using the Rad Prime labeling kit (GIBCO BRL) according to the manufacturer's instructions. Membranes were hybridized with the *hva1* probe according to standard procedures (Sambrook et al. 1989). Hybridized membranes were subsequently analyzed by autoradiography using X-ray film (Kodak) at –80 °C.

Confirmation of transgene expression at the transcriptional level was performed by northern blots. Total RNA was isolated from young leaves of oat plants (transgenic and non-transgenic) using the TRIZOL Reagent (GIBCO BRL) according to the manufacturer's instructions. Aliquots of RNA (10 μg) were fractionated in 1.2% agarose–formaldehyde denaturing gels according to Sambrook et al. (1989) and blotted onto Hybond-N nylon membranes (Amersham-Pharmacia Biotech). Transcripts of *hva1* were analyzed with a standard Northern-blotting method (Sambrook et al. 1989) using the ^{32}P labeled *hva1*-coding sequence as a probe.

Western analysis for HVA1

Confirmation of HVA1 protein production was performed by western blots. Protein isolation, western blotting and immunodetection protocols were as per Xu et al. (1996). Briefly, about 0.1 g of fresh leaf tissue from each sample was pulverized in liquid nitrogen and extracted in 0.2 ml of protein extraction buffer [50 mM sodium phosphate (pH 7.0), 10 mM EDTA (Ethylenediaminetetraacetate], 0.1% (v/v) Triton X-100, 0.1% (w/v) Sarkosyl, 10 mM of β-mercaptoethanol and 10 mM PMSF (Phenylmethylsulfonyl fluoride). Total protein concentration in each sample was measured by the Bradford method (Bradford 1976) using the Bio-Rad protein assay reagent (Bio-Rad). One hundred micrograms of total soluble protein from each sample (transformed or non-transformed) were loaded into each lane of 12% SDS polyacrylamide gels. After electrophoresis, the proteins were electroblotted onto a nitrocellulose membrane (Amersham-Pharmacia Biotech) using a Semi-Dry Transfer Cell (Bio-Rad) following the manufacturer's instructions. The Western blots were incubated with primary antiserum raised against HVA1 protein and secondary antiserum (alkaline phosphatase conjugated anti-rabbit IgG, Sigma-Aldrich). The membrane was developed in alkaline phosphatase buffer (100 mM Tris-base, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.8) containing 0.33 mg/ml of NBT (Nitro blue tetrazolium) and 0.16 mg/ml of BCIP (5-bromo-4-chloro-3-indolyl phosphate).

Analysis of transgenic plants under water-deficit and salt-stress conditions

The performance of transgenic plants expressing the *hva1* gene was evaluated under mannitol and salt-stress conditions using R2 Ogle oat plants from five independent transgenic lines (Ogle BRA-82, Ogle BRA-17, Ogle BRA-8, Ogle BRA-19 and Ogle BRA-41). Seeds of non-transgenic plants were used as controls in all stress tolerance experiments. Fifty R2 seeds from each transgenic line and non-transgenic line were surface-sterilized and germinated in the dark at 25 °C on half-strength MS basal medium ($\frac{1}{2}$MS) containing 1% sucrose and 15 mg/l of glufosinate ammonium (for transgenic lines), and without glufosinate ammonium (for non-transgenic plants), for 4 days. Seedlings were then transferred to $\frac{1}{2}$MS plus 100 mM of NaCl salt medium and $\frac{1}{2}$MS plus 200 mM of mannitol medium and grown under light at 25 °C. These experiments were replicated four times. The response of young seedlings to stress conditions was analyzed after 6 days. The data was collected and plants were transferred to a soil mixture composed of 1:1 (v/v) peat:perlite for further growth and development in the greenhouse. Data was analyzed by analysis of variance (PROC ANOVA, SAS Institute 1985). Means were separated by using Tukey's Studentized range test at a 95% confidence level.

Results

Plant transformation

The transformation results with pBY520 and pAct1-D from eight independent experiments for three oat cultivars are presented in Table 1. Bombarded shoot-tip clumps showed excellent growth without selection for 1 month (Fig. 2a). Dissecting, dividing and subculturing of the growing shoot-tips on different selection media resulted in the selection of putatively transformed shoot-tip

Table 1 Summary of co-transformation experiments with pBY520 + pAct1-D

Cultivars	No. of original shoot clumps	No. of shoot-tips	No. of independent events[a]	Transformation frequency (%)[b]	Relative efficiency (%)[c]	GUS+/bar+/ hva1+[d]
Ogle	16	275	6	37.5	2.2	4/6/6
	15	354	11	73.3	3.1	7/11/11
	16	340	13	81.2	3.8	9/13/13
Pacer	16	295	9	56.25	3.1	6/9/9
	15	323	11	73.3	3.4	8/11/11
	18	284	15	83.3	5.3	9/15/15
Prairie	17	352	12	70.5	3.4	6/12/12
	12	261	10	83.3	3.8	4/9/9

[a] The number of independent events was the total number of independent glufosinate resistant clumps obtained at the end of the 1st month of selection
[b] Transformation frequency after the 1st month of selection measured as glufosinate resistant clumps divided by the total number of bombarded shoot tip clumps, ×100
[c] Relative efficiency of stable transformation measured as the total glufosinate resistant clumps divided by the total number of bombarded shoot tips, ×100
[d] shows the number of independent clumps with each gene

clumps. The transformation frequency after the first month of selection was 64% for Ogle, 70% for Pacer and 77% for Prairie (Table 1). Surviving shoot-tip clumps were used for shoot regeneration after 2–3 months of selection (Fig. 2b). Transgenic plants grew normally in a greenhouse similar to non-transgenic plants and showed fertility (Fig. 2c). The relative efficiency of stable independent transformation events was 3% for Ogle, 4% for Pacer and 3.6% for Prairie (Table 1). The cointegration efficiency for unlinked genes (*bar* and *gus*) was 61.6% compared to 100% efficiency for linked genes (*bar* and *hva1*) in all three cultivars.

Table 2 Segregation of herbicide resistance in the R1 generation

Transformants	Resistant	Sensitive	Expected ratio	χ^2	*P**
Ogle BRA-8	30	12	3:1	0.27	0.60
Ogle BRA-17	70	20	3:1	0.37	0.54
Ogle BRA-19	60	15	3:1	1.00	0.32
Ogle BRA-41	89	26	3:1	0.33	0.56
Pc.BA-4	96	30	3:1	0.09	0.75
Pc. BA-6	84	30	3:1	0.10	0.74
Pc.BA-10	159	61	3:1	0.87	0.35
Pc.BA-11	49	18	3:1	0.12	0.72

*$P < 0.05$

Segregation analysis in R0, R1 and R2 progenies

R0, R1 and R2 progenies were analyzed for the segregation of transgenes by spraying with 1% Ignite to detect *bar* gene expression. The segregation of R1 progeny 10 days after spraying with 1% Ignite is shown in Fig. 2d. Most of the analyzed plants showed normal (3:1) Mendelian inheritance (Table 2).

Histochemical localization of GUS in transformants

Histochemical assays of GUS activity in different tissues and organs of R0 transformants are shown in Fig. 2. One month after selection, chimeric clusters of shoots were produced (Fig. 2e). After the selection regime an increase in GUS activity was observed in the meristematic areas of shoot-tip clumps. A series of free-hand cross-sections of transgenic plants from the greenhouse revealed intense GUS activity in the vascular systems of leaves, stems and roots, and in florets, anthers and stigmas (Fig. 2f and g).

A change was observed in GUS staining intensity in florets during pollination (Fig. 2h). GUS staining in stigmas was more intense 1-h after pollination (Fig. 2hB) as compared to before pollination (Fig. 2hA). The level of GUS activity was unchanged or reduced in stigmas 1-week after pollination compared to before pollination (Fig. 2hC). GUS activity strongly increased in ovaries after pollination.

Molecular analyses of transgenic plants

Southern analysis of R0, R1 and R2 transgenic plants for *hva1* using the single-cutting restriction enzyme (*Hin*dIII) and a double digest (*Hin*dIII-*Bam*HI) confirmed the stable integration of the *hva1* gene without rearrangements into the oat genome (Fig. 3a, b).

Transcripts of *hva1* and the 26-kDa HVA1 protein were found in R0, R1 and R2 transgenic plants (Fig. 4a, b). About 0.01–0.1% of total soluble protein was HVA1 protein. The concentration of HVA1 protein was comparatively higher in young seedlings (data not shown).

Stress-tolerance in HVA 1 expressing R2 plants

Data collected 6 days after in vitro salt and mannitol treatments is shown in Table 3. The average rate of

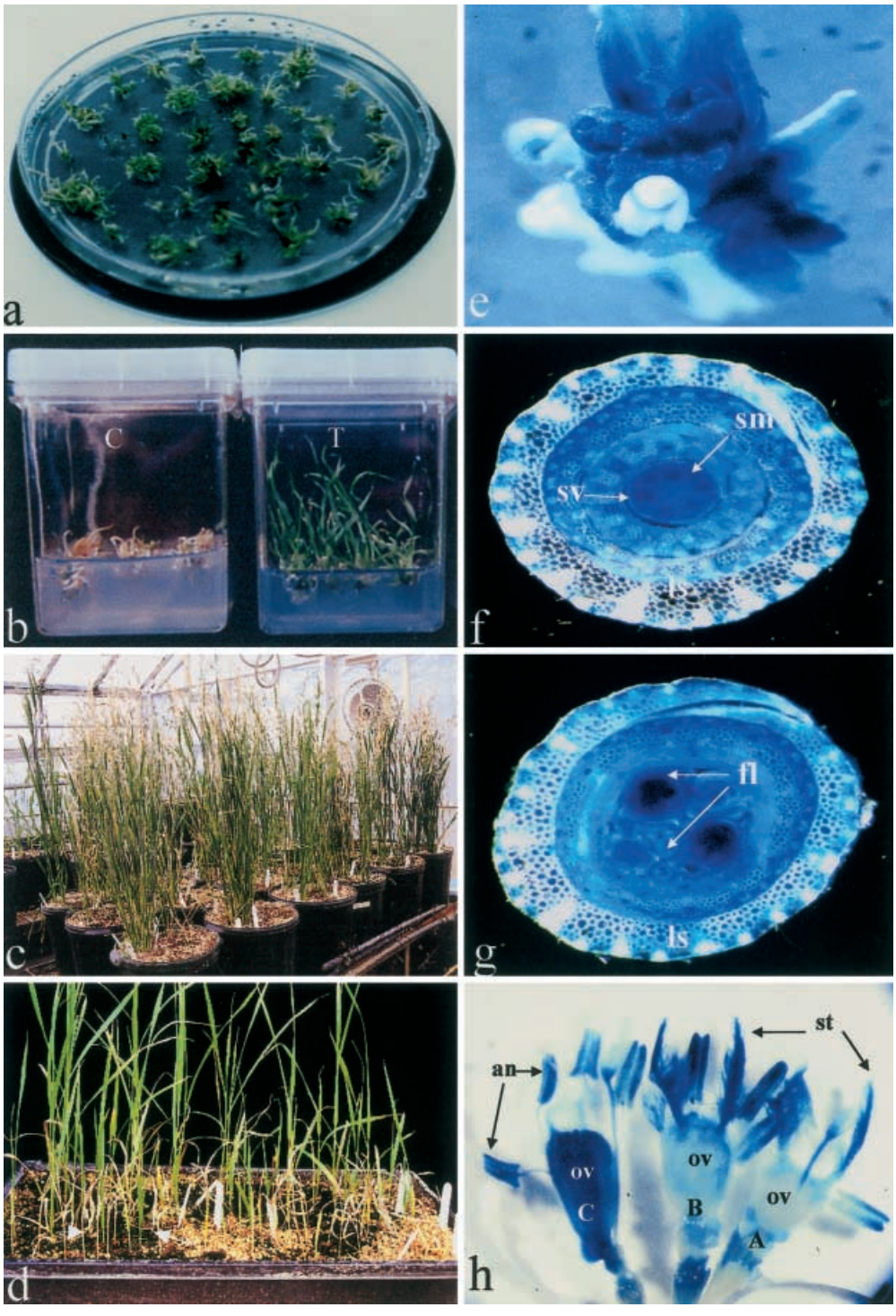

Fig. 2a–h Production of transgenic oat plants and GUS expression in tissues and organs of transgenic plants. **a** Multiplication of multiple shoot cultures 2 weeks after bombardment without selection. **b** Regeneration of transgenic shoots from multiple shoot cultures on selection medium, (*C*) untransformed, (*T*) transformed. **c** Fertile plants matured in the greenhouse. **d** Segregation of R1 after spraying with 1% Ignite. **e** GUS expression in chimeric multiple shoot clumps. **f** Cross-section of leaves and a stem proximal to the shoot tip after GUS staining: (*sm*) stem, (*sv*) stem vascular bundle, (*ls*) leaf sheath. **g** Transverse section showing leaf (*ls*), and florets (*fl*) after GUS staining. **h** GUS expression in immature flowers (*A*) before pollination, (*B*) 1-h after pollination, (*C*) 1-week after pollination: (*an*) anther, (*st*) stigmas, (*ov*) ovary

growth of transgenic seedlings was relatively slower under stress conditions as compared to the transgenic or non-transgenic seedlings grown under non-stress conditions. All transgenic lines showed increased growth rates, in comparison to similarly treated controls, indicating their tolerance to osmotic conditions compared to non-transgenic plants. Almost 80% of non-transgenic plants that were grown under stress conditions were wilted, as compared to 10% wilting in transgenic plants. Different transgenic lines showed different levels of tolerance to salt and mannitol. Overall, we observed 36–49% increases in shoot length, 25–90% increases in root length, and 6–38% increases in the fresh weight of transgenic lines compared to non-transgenic plants analyzed under salt-stress conditions (Table 3). Similarly, we observed 60–88% increases in shoot length, 38–63% increases in root length, and 23–62% increases in the fresh weight of transgenic lines compared to non-trans-

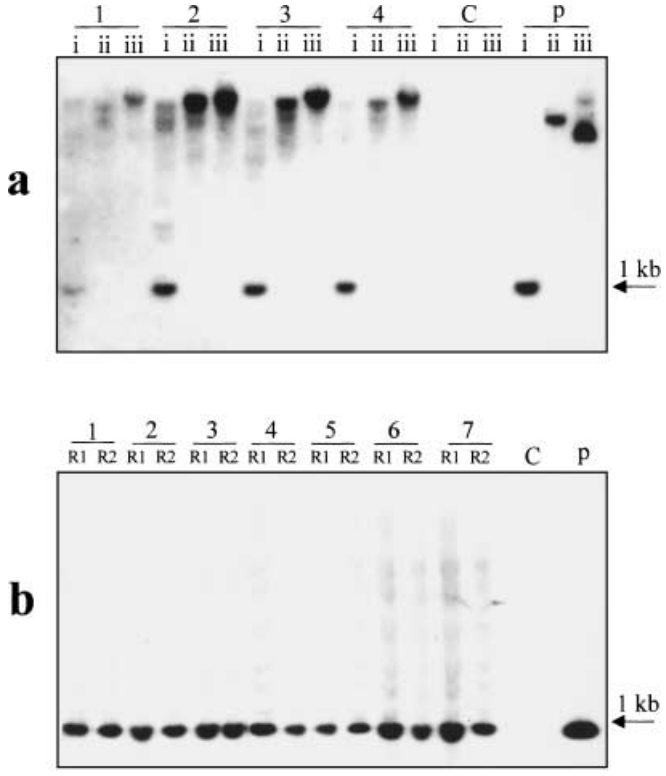

Fig. 3a, b Southern-blot analysis of transgenic oat plants. **a** R0 transgenic oat plants. *Lanes 1–4*: Pc.BA-4, Pc.BA-10, Ogle BRA-82 and Ogle BRA-19 respectively. **b** R1 and R2 transgenic oat plants digested with *Hin*dIII-*Bam*HI. *Lanes 1–7*: Pc.BA-4, Pc.BA-10, Ogle BRA-82, Ogle BRA-17, Ogle BRA-8, Ogle BRA-19 and Ogle BRA-41 respectively. (i) DNA digested with *Hin*dIII-*Bam*HI, (ii) DNA digested with *Hin*dIII, and (iii) Undigested DNA; (*C*) Untranformed, (*p*) Plasmid BY520

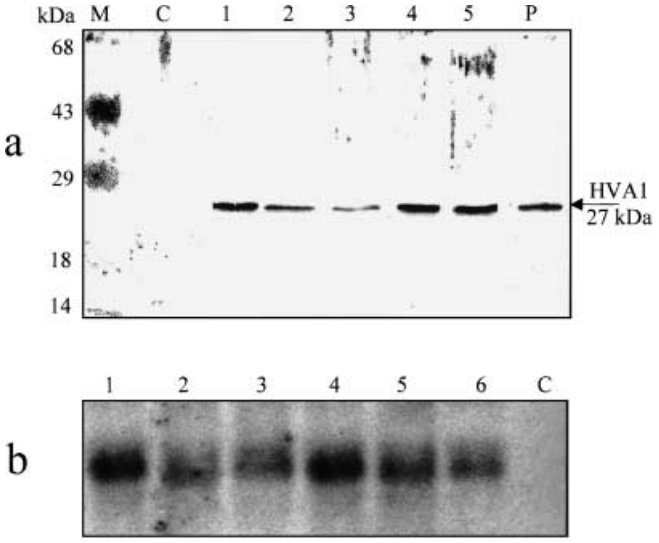

Fig. 4 Expression of *hva1* in transgenic oat plants. **a** Western-blot analysis of HVA1 in R2 progeny. **b** Northern-blot analysis of *hva1* in R2 progeny. *Lanes 1–5*: Ogle BRA-82, Ogle BRA-17, Ogle BRA-8, Ogle BRA-19 and Ogle BRA-41 respectively. (*C*) Protein from non-transgenic oat plant. (*P*) Purified HVA1 protein

genic plants analyzed under mannitol-stress conditions (Table 3). On average, transgenic plants showed 37% more tolerance to salt-stress conditions, and 60% more tolerance to mannitol-stress as compared to non-transgenic plants. Transgenic lines Ogle BRA-82, Ogle BRA-17, Ogle BRA-19 and Ogle BRA-41 showed significantly higher ($P < 0.05$) growth as compared to non-transgenic plants (Table 3).

Linear shoot growth was retarded in control plants under in vitro salt- and mannitol-stress conditions. Upon transfer of transgenic and non-transgenic plantlets from stress-containing media to soil in the greenhouse, without adding any salt or reducing the irrigation, 100% of the transgenic plants survived while only 10% of the non-transgenic plants survived in the greenhouse.

Discussion

Seed germination, root penetration and the growth of crop plant seedlings are generally poor in saline soils (Murty et al. 1984; Verma and Yadava 1986). However, this adverse effect of saline soil can be reduced by growing stress-tolerant genotypes, produced through conventional breeding or genetic engineering.

Table 3 In vitro effect of salt- and mannitol-stress on the growth of young seedlings of transgenic oat cultivars[a]

Lines	Shoot length			Root length			Fresh weight		
	MS cm	MS + NaCl cm	MS + mannitol cm	MS cm	MS + NaCl cm	MS + mannitol cm	MS g	MS + NaCl g	MS + mannitol g
Non-transformed	10.67 ± 0.45	6.26 ± 1.1	5 ± 1.2	4.02 ± 0.45	2 ± 0.6	2.4 ± 0.6	0.2 ± 0.01	0.16 ± 0.02	0.13 ± 0.02
Ogle BRA-82	12.23 ± 0.52[b]	9.05 ± 0.5[b]	9 ± 0.56[b]	5.8 ± 0.88[b]	3.3 ± 0.48[b]	3.9 ± 0.52[b]	0.31 ± 0.025[b]	0.17 ± 0.13	0.20 ± 0.16[b]
Ogle BRA-17	11 ± 1	7 ± 0.64	9 ± 0.7[b]	5.5 ± 0.7[b]	2.5 ± 0.4	3.4 ± 0.3	0.25 ± 0.03	0.17 ± 0.17	0.20 ± 0.03[b]
Ogle BRA-8	10.8 ± 0.7	9.3 ± 0.4[b]	8 ± 1	3.64 ± 0.6	2.9 ± 0.3	3.3 ± 0.4	0.22 ± 0.02	0.20 ± 0.01	0.16 ± 0.02
Ogle BRA-19	11 ± 0.8	8.5 ± 0.9	9.4 ± 0.4[b]	3.9 ± 0.4	3.8 ± 0.6[b]	3.8 ± 0.5[b]	0.16 ± 0.02	0.22 ± 0.02[b]	0.21 ± 0.01[b]
Ogle BRA-41	12.3 ± 0.9[b]	8.5 ± 0.8	9.1 ± 0.63[b]	4.3 ± 0.9	2.7 ± 0.4	3.6 ± 0.25[b]	0.28 ± 0.04[b]	0.22 ± 0.03[b]	0.21 ± 0.01[b]

[a] Data was collected 10 days after seed germination: 4 days in selection medium ($\frac{1}{2}$ MS + 15 mg/l Glufosinate) and 6 days in stress ($\frac{1}{2}$ MS + mannitol and $\frac{1}{2}$ MS + NaCl) and non-stress medium ($\frac{1}{2}$ MS)

[b] Showed significant differences in stress tolerance compared to non-transgenic plants as determined by Tukey's Studentized Range Test, $P < 0.05$

We transformed three oat cultivars with the *hva1* gene for osmotic tolerance and observed stable expression of transgenes in different tissues and organs of transgenic plants. We obtained a high transformation frequency (Table 1) using shoot meristem cultures. These meristematic shoot cultures are highly regenerative and capable of fast in vitro multiplication, and they are excellent target tissues for genetic transformation. Shoots from these transformed meristems showed high fertility and normal sexual transmission of transgenes to their progenies. Histochemical assays of GUS reveal constitutive expression of the Act1 promoter in different tissues and organs of oat, similar to the expression patterns observed in maize and rice (Zhang et al. 1991; Zhong et al. 1996).

Seed germination and early seedling growth are critical stages in crop production, as they indirectly affect crop stand density and grain yield (Gelmond 1978). We tested seedling growth rate, instead of testing the seed germination of HVA1-expressing oat plants in salt- or mannitol-containing media, because the adverse effect of salinity is more pronounced on the growth rate of young seedlings rather than on the rate of seed germination (Murty et al. 1984; Verma and Yadava 1986).

Addition of mannitol to soil or irrigation-water causes plant symptoms similar to water-deficit because it affects the availability of water to plants by increasing the osmotic pressure outside of cells (Rumpho et al. 1983). In our experiments using NaCl and mannitol, transgenic plants maintained a higher growth rate than non-transgenic plants, under both stresses. There were significant differences in wilting, the death of old leaves, and the necrosis of young leaves of non-transgenic versus transgenic plants expressing HVA1. This indicates that the presence of the HVA1 protein in transgenic oat plants confers increased in vitro tolerance to salt and mannitol stresses.

Transgenic lines that expressed HVA1 at moderate and high levels showed increased tolerance to salt and mannitol compared to non-transgenic lines or low-expressing transgenic lines (Table 3; Fig. 4: lanes 1, 2, 4 and 5). The performance of transgenic plants to stress tolerance was directly correlated to the expression level of the HVA1 protein in plants.

Our results on the production of salt- and mannitol-tolerant transgenic oat plants expressing *hva1* in a relatively cultivar-independent manner may become useful for producing salinity and/or drought-tolerant varieties. Our results demonstrated that LEA proteins might play a role in the protection of plants under water- and salt-stress conditions. Therefore, the LEA gene *hva1* can be successfully used for genetic improvement of crops for osmotic tolerance.

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