WHEAT TRANSFORMATION WITH *HVA1* GENE BY MICROPROJECTILE BOMBARDMENT

by

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ABSTRACT

To improve drought tolerance, a gene encoding a late embryogenesis abundant (LEA) protein, *HVA1*, from barley (*Hordeum vulgare* L.) was introduced into winter wheat Jagger (hard red winter wheat) and Lakin (hard white winter wheat). The gene construction, containing *HVA1* gene driven by the rice *Act1* promoter and the selectable marker gene, *bar*, under the control of CaMV35S promoter, was delivered to wheat embryogenic calli by means of microprojectile bombardment. One transgenic wheat plant of Jagger was obtained in this study. This plant survived in medium containing 5 mg/l ammonium glufosinate during the tissue culture processes and has normal morphology. The plant tested positive for the PCR analysis of *bar* gene and was resistant to 0.1% (v/v) herbicide Liberty™. Southern blot analysis showed the integration of *bar* and *HVA1* gene into the genome of this plant. The 27 kDa of *HVA1* protein also was detected in this plant as revealed by Western blot analysis.

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INTRODUCTION

Wheat is one of the most important cereals in the world. Approximately, 24 millions hectares (60 million acres) of wheat are planted in the U.S. and about 54.5 million tons (2 billion bushels) are harvested each year. The United States is responsible for up to 30% of total world wheat exports, although it accounts for only 11% of wheat production worldwide. Wheat is the single most important source of plant protein in the human diet in the U.S..

Wheat productivity is limited by environmental stresses. Stresses, external conditions that adversely affect growth, development, and productivity, can be biotic, i.e. caused by organisms, or abiotic, imposed by an excess or deficit in the physical or chemical environment (Buchanan et al., 2000). Among abiotic stresses, drought and shortage of irrigation water are the major problems affecting crop production worldwide. According to the United Nations resolution, water will become an increasingly scarce natural resource in the next century (www.ppi.biology.queensu.ca/Technologies.htm). Over the last two decades, a search for genes that can beneficially impact drought tolerance in major crops has been made. Many genes that respond to drought in crop plants have been identified but none have been demonstrated to directly influence drought tolerance. (www.csrl.ars.usda.gov/pswc/ research/accom.html)

I. Plant responses to water deficit

Responses of plants to water deficit are influenced by the amount and rate of water lost and the duration of the stressed condition. Plant water deficit is caused by different stresses including drought, salinity, and temperature. It occurs when the rate of

transpiration exceeds the rate of water uptake. However, some of the common developmental processes, such as seed development, also can cause such water deficit. Ability to withstand the stresses, tolerance or a mechanism that permits avoidance of the negative effects of the imposed stress, imply resistance of the plants to water deficit . Plants must withstand multiple abiotic and biotic stresses in an unfavorable environment.

The recognition of the stress is the first step in regulating the water deficit response. Such recognition includes loss or decrease of turgor, change in cell volume or membrane area, loss of membrane 'stretch', change in water activity or solute content, and alteration in cell wall-plasma membrane connections or protein-ligand interaction. Many aspects of cellular water loss may be measured by these stress recognition mechanisms. Following the cellular perception of water loss is the activation of the signal transduction pathway which leads to the induction of specific genes. Under the same conditions or with the same cell types, not all stress-specific genes are induced and it appears that several different signaling mechanisms are involved. During water deficit many changes in gene expression occur. In addition to genes that are directly concerned with this stress, other genes also may be induced during this period (Bray, 1997). However, functions and mechanisms of many stress-responsive genes are still not clear (Xu et al., 1996).

Generally, the response to water deficit involves mechanisms to avoid water loss, to repair damage and to protect the cellular machinery. To avoid water deficit, plants synthesize osmolytes, compatible solutes such as sugar alcohols, special amino acids and glycine betaine that can accumulate to high levels without disrupting protein function. During the response to repair damage due to water deficit, many proteins are

induced, including ubiquitin, chaperones and proteases. These proteins may be involved in the recovery of proteins or in the degradation of defective or damaged proteins. However, it is still unclear whether the induction of genes encoding these proteins is to repair damage caused directly by reduced water content, or if they accumulate to heal damage caused by a secondary stress or to restrict pathogen invasion. In addition to these mechanisms, plants protect the cellular machinery of developing embryos by accumulating many hydrophilic globular proteins during seed maturation. These proteins were grouped and called 'late embryogenesis abundant' (LEA) proteins (Bray, 1997).

II. LEA Proteins (Late Embryogenesis Abundant Proteins)

Among drought tolerance genes studied so far, the LEA protein genes have shown promise as protectants against drought stress. These genes are under the control of the abscisic acid-regulated promoter elements (Curry et al., 1993). LEA genes are a group of genes that are expressed in embryos during the late stages of embryogenesis before dessication and in vegetative tissues when plants are subjected to drought condition (Zhang et. al., 2000, Sivamani et al., 2000). Examples of LEA proteins are LE25, a group 4 LEA protein from tomato (*Lycopersicum esculentum L.*), which improved resistance to high salinity and freezing when expressed in yeast (*Saccharomyces cerevisiae*) (Imai et al., 1996) and Em, a wheat LEA protein, which functioned as an osmoprotective molecule in yeast (Swire-Clark and Marcotte, 1999). However, not all LEA proteins are always correlated with stress tolerance (Xu et al., 1996). LEA proteins are classified into 3 groups based on the amino acid sequence homologies. They were first characterized in cotton (Dure, 1981). Recently,

characterization of LEA proteins from other plant species has been achieved (collated by Dure, 1992). LEA proteins are characteristically highly hydrophilic and are soluble in boiling water. These characteristics suggest that LEA proteins are involved in the protection of plant cells from dehydration. Accumulation of LEA proteins coincides with freezing and desiccation tolerance in plants (Zhang et al., 2000). Additional evidence supporting a role for LEA proteins in stress tolerance is demonstrated by the correlation between LEA gene expression or LEA protein accumulation and stress tolerance in a number of plants (Xu et al., 1996). For instance, the accumulation of a high level of group 3 LEA proteins was correlated with tissue dehydration tolerance in severely dehydrated wheat seedlings (Ried and Walker-Simmons, 1993). In several salt-tolerant Indica rice cultivars (*Oryza sativa* L.), the levels of group 2 LEA proteins (also known as dehydrins) and of group 3 LEA proteins in roots were significantly higher when compared to salt-sensitive cultivars. Further these proteins are induced by ABA and salt stress in salt-tolerant cultivars suggesting a role in stress tolerance (Moons et al., 1995). However, the exact function and physiological roles of LEA proteins are still unknown (Xu et al. 1996).

III. Barley HVA1 gene

A barley (*Hordeum vulgare* L.) *HVA1* gene was classified into group 3 LEA proteins as an ABA-inducible gene. It was abundant or exclusively produced in the aleurone layers and embryos during the late seed development, correlating with desiccation of seeds (Hong et al., 1988). The barley *HVA1* gene and wheat p*MA2005* gene share a highly similar structural gene organization. These two genes are closely

related to the carrot Dc3 and cotton D-7 genes (Straub et al., 1994). Although, the open reading frame of *HVA1* gene encodes a 22 kDa protein, the products from *in vitro* transcription and subsequent translation comigrate on SDS gel as a 27 kDa polypeptide. The size discrepancy is probably due to the high content of the basic amino acid, lysine (Hong et al., 1988). Group 3 LEA proteins of rice, wheat, and barley have similar electrophoretic mobilities (Xu et al., 1996). The regions of homology of group 3 LEA proteins commonly contain a repeat of an 11-mer amino acid motif with the consensus amino acid sequence TAQAAKEKAGE. This region may form an amphiphilic α-helical structure and may allow dimerization of the polypeptides. Due to the arrangement of charged amino acids within this motif, a function for this group of proteins in sequestering ions that will accumulate under dehydration conditions was suggested (Zhang et al., 2000).

Evidence is still lacking to demonstrate the precise mode of action of *HVA1* gene under drought conditions. "It is believed that *HVA1* may affect cell structure globally, such as, by binding to proteins in the cytoskeleton, thus changing the cell permeability or integrity. It may also affect cellular metabolism and thus influence the production of ATP via glycolysis or photosynthesis" (Bajaj et al., 1999).

Accumulation of *HVA1* protein in transgenic rice showed increased growth rates, delayed damage symptoms and improved recovery (Xu et al., 1996). Increase in biomass and water use efficiency were demonstrated in HVA1 transgenic spring wheat (Sivamani et al., 2000)

IV. Wheat Transformation

Due to the limitations of classical breeding, such as genetic incompatibility, time constraints, and labor requirements, crop improvement by genetic transformation seems to be the method of choice. However, monocotyledonous plants, cereal crops in particular, have lagged behind dicotyledonous plants in ease and efficiency of transformation (Weeks et al., 1993). Gene transformation provides a method to incorporate genes from distantly related or cross-incompatible species into the desired host leading to a permanent change of the genetic constitution bypassing a sexual cross. Available approaches for genetic transformation include microprojectile bombardment (Sanford et al., 1987; Christou et al., 1988), *Agrobacterium*-mediated transformation (Hiei et al., 1994; Cheng et al., 1997), microinjection (Crossway et al., 1986; Reich et al., 1986), DNA uptake by electroporation (Toriyama et al., 1988; Zhang et al., 1988; Zhang and Wu, 1988; Shimmamoto et al., 1989; Datta et al., 1990; Peng et al., 1992), polyethyleneglycol (PEG)-mediated transfer of genes (Marsan et al., 1993) by protoplast-mediated transformation, silicon carbide fibre-induced DNA uptake (Wang et al., 1995), laser-mediated DNA uptake (Guo et al., 1995). Some approaches gradually have been phased out because of their limitations and disadvantages. Difficulty in regenerating fertile plants from the protoplasts of cereals other than rice (Fromm et al., 1990) after long period in tissue culture is an example. Electroporation and PEG–mediated protoplast transformation (Li et al., 1993) cannot be achieved in some plant species due to the so-called genotype dependence (He et al.,1994). To date, microprojectile bombardment and *Agrobacterium*-mediated transformation are the two main methods which are being used and developed in most transformation laboratories.

Advantages of *Agrobacterium*-mediated transformation include the typical insertion of one or a few copies of the transgenes, often into actively transcribed regions of the plant genome, and the potential to transfer large pieces of DNA. However, *Agrobacterium* strains are not prone to infect most monocotyledonous species efficiently, and thus other alternatives are preferred (Hammond et al., 1999).

Thus, for the most recalcitrant cereal species including wheat, microprojectile bombardment is the most used technique for gene transformation to date. Many transgenic wheat plants have been recovered through this method. (Vasil et al., 1993; Becker et al., 1994; Chen et al., 1998a; Blechl and Anderson, 1996; Altpeter et al., 1996; Barro et al., 1997; Bliffeld et al., 1999). Co-transformation of multiple plasmids is carried out by the microprojectile bombardment method (Hadi et al., 1996).

The production of genetically engineered crops with resistance or tolerance to environmental extremes is made possible by the recent advances in plant biotechnology. More than 20 genetically engineered crops are now available commercially including cotton, corn, and soybeans with herbicide and insect resistance traits. However, there is currently no genetically-engineered wheat on the market. The widespread use of transformation technology is still impeded by many parameters. The procedure is not efficient, requires a lot of expertise for routine use, is time consuming, and is feasible with only some species.

In order to screen the transformed plants from a large number of tissues that are subjected to gene transformation methods, the *bar* gene is the most commonly used selectable marker gene in wheat transformation research. This gene is a bialaphos-resistance gene from the bacterium *Streptomyces hygroscopicus* and encodes

phosphinothricin (PPT) acetyltransferase (PAT). This enzyme acetylates the free NH_2 group of PPT and thus prevents autotoxicity in the producing organism (Murakami et al., 1986). Bialaphos is a tripeptide antibiotic which consists of PPT, an analogue of L-glutamic acid, and two L-alanine residues. PPT inhibits glutamine synthetase (GS) causing rapid accumulation of ammonia which leads to death of the plant cells (Tachibana et al., 1986). For wheat transformation research, 5 mg/l bialaphos is preferred for selecting the transformed calli (Chen et al., 1998a).

Because wheat is a low-price crop and its genome is so large (16 billion bp), wheat improvement by biotechnology is progressing very slowly compared to other crops like rice or cotton. Despite significant advances in transformation of other cereals, routine transformation of many elite wheat cultivars is not yet possible. Most achievements in wheat transformation have been made with spring wheats, especially the cultivar 'Bobwhite', even though, in the U.S., spring wheats account for only 35% of total wheat production compared to a value of 65% for winter wheat. Thus, backcrosses of engineered spring wheat to winter wheat are still necessary. Currently, most research on wheat transformation is focused on drought tolerance and fungal disease resistance traits.

The goal of this study was to introduce the drought tolerance gene, *HVA1*, from barley, under the control of the rice *Act1* promoter directly into two winter wheat cultivars, Jagger and Lakin by microprojectile bombardment with the *bar* gene as the selectable marker.

MATERIALS AND METHODS

Plant materials and tissue culture

The hard red winter wheat, Jagger, and the hard white winter wheat, Lakin, were grown in growth chambers at 18 to 22 °C with 16-h photoperiod of approximately 150 μmole/s/m^2 of light intensity.

Immature seeds were harvested 13-15 d after anthesis. Seeds were surface-sterilized for 2 min in 70% ethanol, washed once in sterile distilled water and then soaked in 25% Clorox (containing 2 drops of Tween 20 per 50 ml solution) for 15 min. The Clorox solution was then decanted and seeds were finally washed 5 times (1 min each) with sterile distilled water (http://agad srv.msu. montana.edu/ Weed Trans/steriliz.htm).

Immature zygotic embryos (0.5-1.5 mm diameter) were aseptically removed and placed on the callus-induction medium (Table 1) with the scutellar side up. The cultured embryos were incubated at 24-27 °C during callus formation, osmotic treatment and regeneration. After 5-8 d of culture in the dark, the embryogenic calli were subjected to microprojectile bombardment with a Biolistic® PDS-1000/He (Bio-Rad) gun. Four to six h prior to and 16 h after bombardment, the embryogenic calli were incubated on osmotic medium (Table 1). The bombarded embryogenic calli were then transferred to the callus-induction medium plus 5 mg/l ammonium glufosinate for about 14 d in the dark and then transferred to the regeneration medium (Table 1) plus 5 mg/l ammonium glufosinate.

Table 1. Tissue culture media for wheat transformation

Medium	Composition
Callus induction medium	MS salt and vitamin mixture (Table 2) , 2 mg/l 2,4-D, 100 mg/l casein hydrolysate, 30 g/l sucrose, adjusted to pH 5.7 and added 2.2 g/l Phytagel
Osmotic medium	MS salt and vitamin mixture, 2 mg/l 2,4-D, 30 g/l sucrose, 0.4 M mannitol, adjusted pH to 5.7 and added 2.2 g/l Phytagel
Callus induction medium plus 5 mg/l ammonium glufosinate	MS salt and vitamin mixture, 2 mg/l 2,4-D, 30 g/l sucrose, adjusted pH to 5.7 and added 2.2 g/l Phytagel
Regeneration medium plus 5 mg/l ammonium glufosinate	MS salt and vitamin mixture, 1 mg/l kinetin, 0.5 mg/l IAA, 20 g/l sucrose, adjusted pH to 5.7 and added 2.2 g/l Phytagel
Rooting medium plus 5 mg/l ammonium glufosinate	Half-strength MS salt and vitamin mixture, 15 g/l sucrose, adjusted pH to 5.7 and added 2.2 g/l Phytagel

Table 2. Composition of Murashige and Skoog (MS) medium Salt and Vitamin Mixture, 1962

Component	mg/l
Macronutrients	
NH_4NO_3	1,650.00
KNO_3	1,900.00
$CaCl_2$	332.20
$MgSO_4$	180.70
KH_2PO_4	170.00
Micronutrients	
H_3BO_3	6.20
$MnSO_4.H_2O$	16.90
$ZnSO_4.H_2O$	8.60
Na_2EDTA	37.26
$FeSO_4.7H_2O$	27.80
Trace elements	
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.6H_2O$	0.025
$CoCl_2$	0.025
Vitamins :	
Myo-inositol	100.00
Nicotinic Acid	0.50
Pyridoxine · HCl	0.50
Thiamine · HCl	0.10
Other Component :	
Glycine (Free base)	2.00

Base Cat. No. 10632, Life Technologies.

In the regeneration period, embryogenic calli were cultured under fluorescent lights (16-h photoperiod). Subcultures were made every 2-3 weeks until green shoots developed. Embryogenic calli with green shoots were then transferred to rooting medium plus 5 mg/l ammonium glufosinate (Table 1). The plantlets were grown in the rooting medium until they reached a height of 4-5 cm and developed a strong root system. Then they were transferred to soil and grown to maturity in growth chambers under the conditions applied for the growth of donor plants.

Microprojectile bombardment

Prior to bombardment, microcarriers were prepared according to the instruction manual from the manufacturer (Bio-Rad Laboratories). An aliquot of 40 µl of the stock microcarrier solution (60 mg/ml) was used, followed by adding, in sequence, (under continuous vortexing) 5 µg of plasmid DNA, 250 µl of 2.5M $CaCl_2$, 50 µl of 0.1 M spermidine (free base) and 220 µl of sterile water. The mixture was shaken at 4°C for 15 min and centrifuged at 1,000 x g for 10 min. The supernatant was removed, the particles were then washed with 200 µl of absolute ethanol and finally resuspended in 40 µl of absolute ethanol. Ten µl of particle suspension was used for each bombardment.

Biolistic PDS-1000/He (Bio-Rad) gun was used in this experiment. Microprojectile bombardment were performed at 7,590 kPa (1,100 psi), a 8 and 10 cm particle travel distance, an 8-10 mm gap distance between the rupture disk and the microcarrier, and a 12 mm macrocarrier flying distance.

Confirmation of the presence of transgenes

PCR analysis

The presence of *bar* gene in putative transgenic wheat plants was carried out by using REDExtract-N-Amp™ Plant PCR Kit (Sigma). Samples of leaf disks were collected with a standard paper punch and incubated in 100 µl of Extraction Solution at 95°C for 10 min to extract genomic DNA. The genomic DNA was then diluted with 100 µl of Dilution Solution to neutralize inhibitory substances prior to PCR. Four µl of the DNA extract was then added to the PCR reaction containing a specially formulated Plant PCR and *bar* gene primers. This set of primers was designed from the coding region of the *bar* gene. The sequence of its forward primer was 5'-AAGCACGGTCAACT TCCGTA-3' and of its reverse primer was 5'-GAAGTCCAGCTGCCAGAAAC-3'. The expected size of the amplification product is 394 bp. The PCR profile consisted of a cycle of 5 min at 95°C; 30 cycles of 30 sec at 95°C, 30 sec at 62.8°C, 1 min at 72°C; and final extension for 10 min at 72°C. The PCR products were detected by loading the whole 20 µl PCR reaction into 1% agarose gel containing 0.5 µg/µl ethidium bromide and electrophoresing at 75 V for ~1 h. 1 kb DNA Ladder (Promega) was used as a standard molecular-weight marker.

Southern blotting

Genomic DNA extraction (Maize Miniprep, Dellaporta et al., 1983)

One to two grams of wheat leaf tissue was frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. The powder was then transferred to a 50 ml tube. Fifteen ml of extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, and 10 mM mercaptoethanol) and 1 ml of 20% SDS were added into the tube.

The mixture was shaken vigorously and incubated at 65°C for 30 min. Then, 5 ml of 5 M potassium acetate was added, mixed thoroughly and incubated on ice for 20 min. The mixture was centrifuged at 6,000 rpm. The supernatant was filtered through a Miracloth filter (Calbiochem) and transferred to a clean 50 ml tube. Ten ml of isopropanol was added to the supernatant, mixed and incubated at -20°C for 30 min. The recovered DNA was vacuum-dried and dissolved overnight in 700 µl of preheated $T_{10}E_1$ (10 mM Tris-Cl, pH 7.5; 1 mM EDTA, pH 8.0). Three hundred and fifty µl of phenol was added, mixed, and incubated for 5 min. Then 350 µl of chloroform was added and the mixture was centrifuged at 6,000 rpm for 5 min. The upper layer was collected. An equal volume of chloroform was added to the collected mixture and then the total mixture was centrifuged for 5 min. The upper layer was collected and transferred to a new microcentrifuge tube. Seventy-five µl of 3 M sodium acetate and 500 µl of isopropanol were added and mixed. The DNA was pelleted by centrifugation for 30 s. The DNA was washed in 70% ethanol, dried, and dissolved again in 100 µl $T_{10}E_1$. To estimate the DNA concentration, 1 µl of extracted DNA was treated with 1 µl of 10 mg/ml RNase and electrophoresed in 1% agarose gel containing 0.5 µg/ml ethidium bromide, along with the standards.

DNA digestion, electrophoresis and blot transfer

Twenty µg of wheat genomic DNA was digested at 37°C overnight with *EcoR*V (175 units) or *Hind*III (160 units) restriction enzyme. The digested DNA was then subjected to 1-1.2% gel agarose gel electrophoresis at 35 V for ~ 12 h. After completing electrophoresis, the gel was stained in 300 ml of 1 x TAE (40 mM Tris-acetate; 1 mM

EDTA, pH 8.0) buffer with 15 µl of ethidium bromide (10 mg/ml) for ~ 20 min and photographed under UV light. The DNA fragment was depurinated by soaking the gel in 250 ml of 0.25 M HCl until the dye turned yellow (~15 min), then rinsed with distilled water and neutralizing in 250 ml of 0.4 M NaOH until the dye turned back to blue (~20 min). DNA fragments were then transferred onto positively charged nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech) using the alkaline upward capillary transfer method.

Probe preparation

The 1.0 kb *HVAI* fragment was obtained by digestion of pBy520 (Fig. 1) with *Hind*III and *BamH*I and, in order to obtain the 568 bp *bar* gene fragment, pAHC20 (Fig. 2) was digested with *BamH*I and *Bgl*II. The digested plasmid was electrophoresed in 1% low melting agarose gel (Fisher Biotech) to elute the fragments of interest and purified by Wizard™ PCR Prep DNA purification System Kit (Promega). Fifty to sixty ng of the probes were boiled for 5 min and cooled down on ice. The denatured probes were then labeled with (α-^{32}P) dCTP using Prime-a-Gene Labeling System (Promega). The 50 µl labeling reaction included probe DNA (20 ng/µl) 3 µl, 10 x primer BSA 2.5 µl, 5 x labeling buffer 10 µl, 1.5 mM dNTP 4 µl, Klenow enzyme 1 µl, (α-^{32}P) dCTP 5 µl and distilled water 24.5 µl. The labeling reaction was incubated at 37°C for 3-4 h or at room temperature overnight. The labeled probes were purified through a Micro Spin™S-400 HR Column (Amersham Pharmacia Biotech).

Prehybridization, hybridization and autoradiography

pBy520

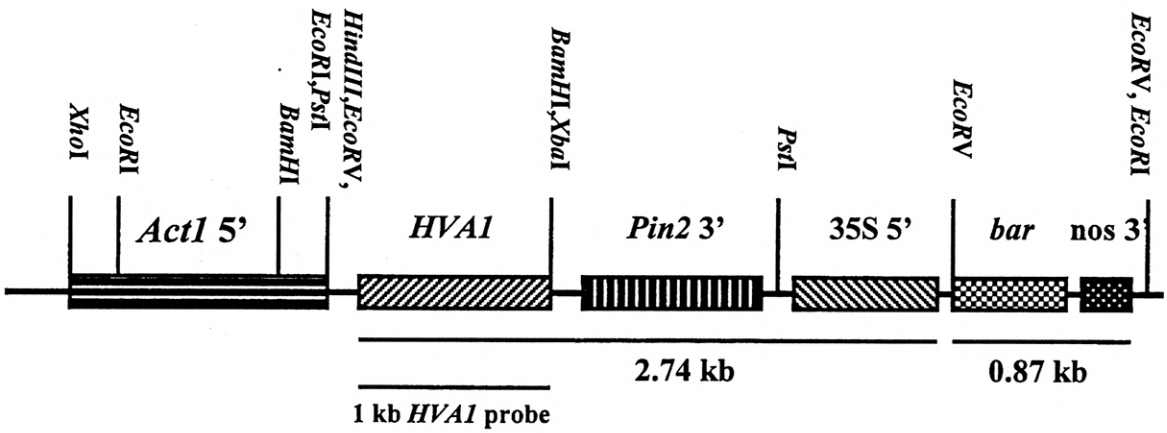

Fig 1. Structure of the plasmid pBy520 for expression of *HVA1 gene*

Expression of the *HVA1* structural gene is regulated by the rice *Act1* promoter and the potato *Pin2* 3'region. The *bar* gene which is regulated by the CaMV35S promoter and the nopaline synthase gene (nos) 3' region serves as the selectable marker. The two gene expression cassettes were cloned into the plasmid pBluescriptIIKS(+). This plasmid was provided by Dr. Ray Wu, Cornell University.

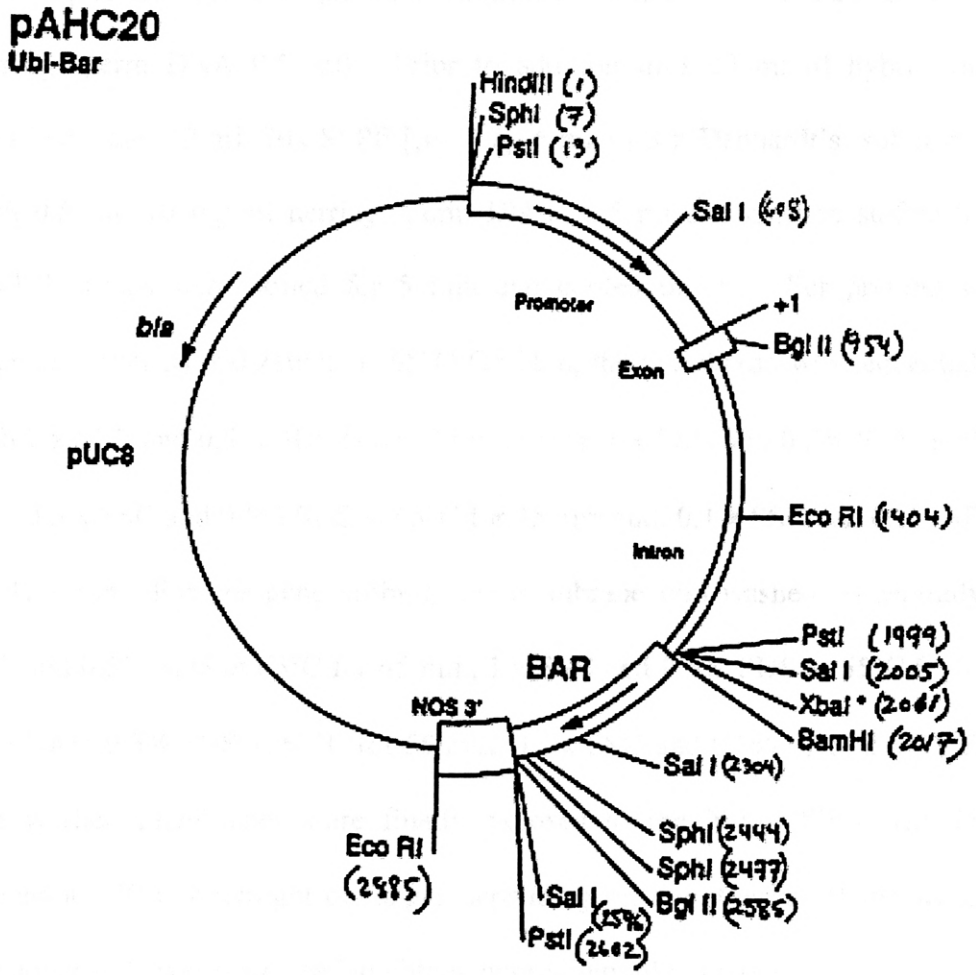

Fig 2. Complete map of pAHC20 vector

Prehybridization of membranes was made at 65°C for ~20 h in a hybridization tube containing 50 ml of prehybridization buffer (distilled water 30.75 ml, 20 x SSPE [pH 7.7] 15 ml, 100 x Denhardt's solution 2.5 ml, 20% SDS 1.25 ml and 10 mg/ml herring sperm DNA 0.5 ml). Prior to addition into 20 ml of hybridization buffer (distilled water 12 ml, 20x SSPE [pH 7.7] 6 ml, 100 x Denhardt's solution 1 ml, 20% SDS 0.5 ml, 10 mg/ml herring sperm DNA 0.5 ml and dextran sulfate 0.5 g), the labeled probes were boiled for 5 min and cooled on ice. For probing with *HVA1* fragment, after hybridization at 65°C for 18 h, the membrane was sequentially washed with 2 x SSC and 0.5% SDS at 65°C for 35 min, 1 x SSC and 0.5% SDS at 65°C for 30 min, 0.5 x SSC and 0.5% SDS at 65°C for 45 min and, 0.1 x SSC and 0.1% SDS at 65°C for 120 min. For *bar* gene probing, the membrane was washed sequentially with 2 x SSC and 0.5% SDS at 65°C for 45 min, 1 x SSC and 0.5% SDS at 65°C for 15 min, 0.5 x SSC and 0.5% SDS at 65°C for 55 min, 0.1 x SSC and 0.1% SDS at 65°C for 15 min. The washed membranes were finally exposed to the X-ray films with intensifying screens at -70°C overnight or longer depending on the strength of the signal. Kodak developer and fixer were used to obtain autoradiographic images.

Test of bar gene expression by Liberty™ application

Liberty™ (18.2% w/v ammonium glufosinate) solution was diluted to 0.1% (v/v) and applied with cotton swabs to segments (2.5-5.0 cm) of selected leaves of putative transformed and nontransformed (control) wheat plants then monitored leaf responses daily.

Analysis of HVA1 gene expression

Protein extraction and determination of concentration

Fresh leaves (150-250 mg) from putative transgenic and control wheat plants were collected and ground into a fine powder using liquid nitrogen. The fine powder was then suspended in 100-150 µl of protein extraction buffer (0.125 M Tris-HCl, pH 6.8, 2.5% SDS, 10% glycerol) in a microcentrifuge tube. Crude protein extracts were incubated at 100°C for 5 min. Supernatants were collected by centrifugation at 13,000 x g for 5 min at room temperature. The protein concentrations of the extracts were determined by the bicinchoninic acid microtiter plate assay (Pierce, IL, USA), using bovine serum albumin (2 mg/l) as a standard.

SDS-polyacrylamide gel electrophoresis

Prior to loading, protein samples were prepared by mixing 350-500 µg of protein extracts with 1/3 volume of 4x gel-loading buffer (0.25 M Tris-Cl pH 6.8, 40% glycerol, 4% SDS, 4% β-mercaptoethanol), heated to 100°C for 5 min and then cooled on ice. Samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a Hoefer Scientific Dual Mini gel apparatus. Samples were separated in 15% SDS-polyacrylamide gels and electrophoresed at 25 mA per gel. Five µl of full range Rainbow molecular weight marker (Amersham Pharmacia Biotech) was used as the molecular weight standard.

Western blotting

After electrophoresis, the proteins were transferred from the polyacrylamide gel

to an Immobilon™-P Transfer Membrane (Millipore) using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad) at 140 mA for 30 min per gel. The membrane was then blocked in 3% (w/v) bovine serum albumin for at least 2 h. After blocking, the membrane was subjected to the first antibody, the rabbit anti-*HVA1* antibody (a kind gift from Dr. Tuan-Hua David Ho of Washington University, St. Louis, MO, and Dr. Ray Wu of Cornell Universtiy), overnight. Prior to use, the *HVA1* antibody was diluted to 1 : 5,000 in 1x TBST (10 mM Tris pH 7.5, 140 mM NaCl and 0.5 mL/L Tween 20). Then the membrane was washed in 1x TBST 5 times with 5 min for each wash continued by immersing the membrane into the second antibody (goat anti-rabbit IgG alkaline phosphatase conjugate, (Bio-Rad)) for ~3 h. The second antibody was diluted to 1 : 3,000 with 1x TBST prior to use. The membrane was then washed 3 times with 1x TBST followed by 2 washes with 1x TBS (10 mM Tris pH 7.5, 140 mM NaCl), for 5 min for each wash. The second antibody was detected using 4-nitroblue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrates supplied in an alkaline phosphatase immunoassay kit from Bio-Rad.

RESULTS

Regeneration efficiency of wheat cultivars used in the experiment

To screen for the regeneration potential of the donor plants, immature embryos of three elite winter wheat cultivars, Lakin, Jagger and Heyne, were cultured under the same conditions that were applied during the transformation processes but without the selection with ammonium glufosinate. After 10 days on the regeneration media, calli of Lakin, Jagger and Heyne gave rise to 87, 55 and 23 %, respectively, green shoots (Table 3). All calli with shoots were then transferred to rooting medium. At the 20^{th} d on rooting medium 73, 29 and 19 % of Lakin, Jagger and Heyne, respectively, regenerated small seedlings. Based on these regeneration efficiencies, in subsequent experiments, Lakin and Jagger were selected as donor plants for the transformation experiments.

Selection, somatic embryogenesis and plant regeneration

With the scutellum-side up, immature embryos of two winter wheat cultivars, (*Triticum aestivum* L., cvs. Jagger and Lakin) were excised from immature seeds under aseptic conditions and cultured on callus induction medium for 5-7 days in dark. The formation of somatic embryogenesis was observed by early cell division activity of competent embryogenic cells contained in the scutella which gave rise to a mass of nodular creamy embryogenic callus within a week of the initiation of cultures. To promote the growth of scutellar calli and inhibit the precocious germination of the original embryos, 2 mg/l auxin, 2,4-D, was supplied in callus induction medium (Vasil et al., 1992).

Table 3. Regeneration efficiency of three winter wheat cultivars

Cultivar	Number of embryos	Number of green shoots observed on 10th day on regeneration medium	Number of seedlings observed on 20th day on rooting medium
Lakin	165	143 (87%)	121 (73%)
Jagger	178	98 (55%)	52 (29%)
Heyne	171	40 (23%)	32 (19%)

Five to seven-day-old embryogenic calli were bombarded with a single plasmid DNA, pBy520 (Fig. 1), or the combination of pBy520 and pAHC20 (Fig. 2) in separate experiments (Table 4). After bombardment, the calli were transferred to the callus induction medium containing 5 mg/l ammonium glufosinate for 2 weeks and subsequently to the regeneration medium for shoot regeneration. After green shoots developed, the calli with shoots were transferred to the rooting medium. To ensure complete inhibition of the regeneration from the nontransformed plants, 5 mg/l ammonium glufosinate was included in regeneration and rooting media through the last period of the tissue culture processes (Fig. 3).

During the screening process with 5 mg/l ammonium glufosinate, the majority of the non-transformed plantlets that survived were strongly retarded in growth and became necrotic or chlorotic. They exhibited poor root development and their leaves gradually turned brown from the tips throughout the whole plants. They stopped growing and died eventually. Although the putative transformed plantlets developed long branched roots and green leaves in the presence of 5 mg/l ammonium glufosinate, their growth was less than that of non-transformed plants growing without the herbicide. Plants that survived the selection but tested negative for PCR analysis and Liberty™ painting were considered escapes.

PCR analysis of bar gene in transformed wheat plants

To detect the presence of *bar* gene, all of the primary putative transgenic plants were analyzed by PCR. Only one of them, Jag#A, yielded the expected 394 bp amplification product while no product was detected in genomic DNA from a

Table 4. Summary of transformation experiments

Bombardment combinations		Jagger			Lakin		
plasmids	Particle travel distance	Number of calli bombarded	Number of seedlings	Number of transgenic wheat plants (% transformation frequency)	Number of calli bombarded	Number of seedlings	Number of transgenic wheat plants (% transformation frequency)
pBy520	8 cm	206	2	1 (0.5)	2,056	2	0 (0)
pBy520	10 cm	164	1	0 (0)	253	0	0 (0)
pBy520 : pAHC20 (1:1 molar mixture)	10 cm	1,000	0	0 (0)	273	1	0 (0)
PBy520 : pAHC20 (4:1 molar mixture)	10 cm	633	0	0 (0)	112	0	0 (0)

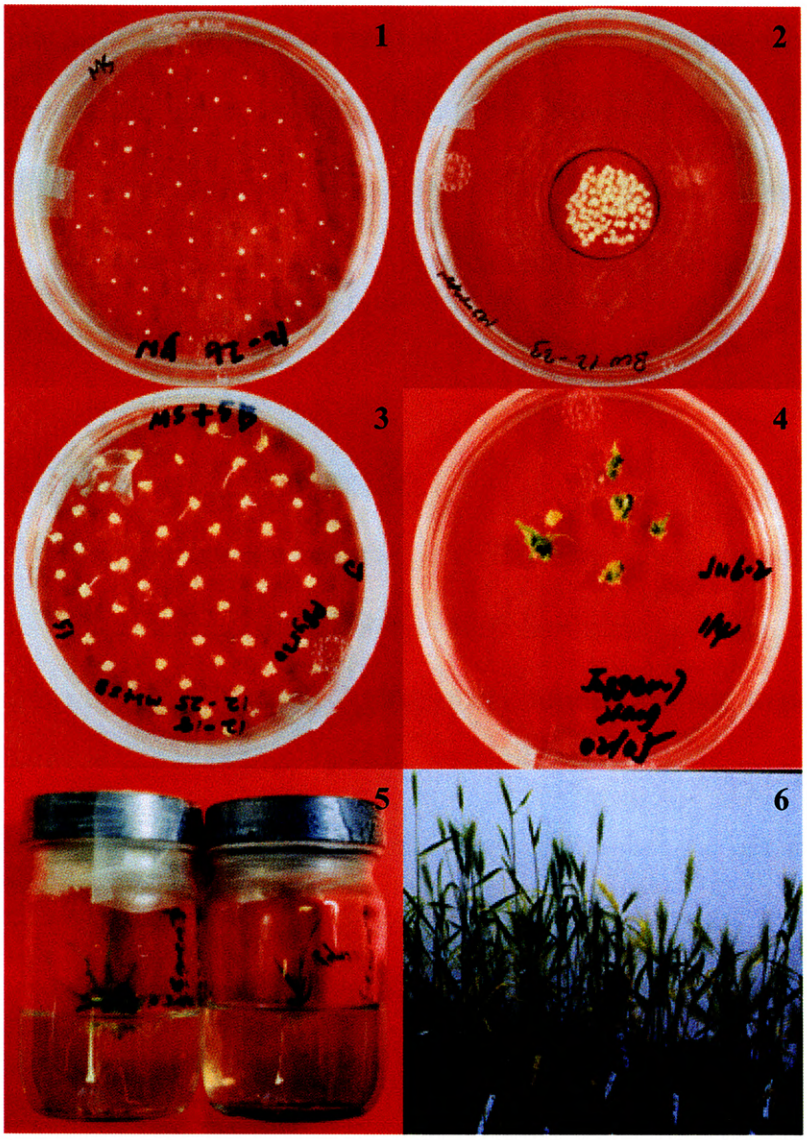

Fig 3. From immature embryos to mature plants via tissue culture processes

(1)Immature embryos; (2)bombarded calli on osmotic medium; (3)calli on the selection medium (5 mg/l ammonium glufosinate); (4)young shoots on the regeneration medium; (5)seedlings in rooting medium; (6)mature wheat plants

nontransformed plant (which served as a negative control) and other putative transformed plants (presumed to be escapes). To confirm the presence of *bar* gene among all tillers of the plants, genomic DNA from leaves of every tiller of putative transformed plants were analyzed. The expected 394 bp amplification products were obtained with DNA from all tillers of Jag#A plant. The results indicated that this plant was not chimeric (Fig. 4).

Resistance of plants to Liberty™ application

To determine the expression of *bar* gene which was under the control of CaMV 35S promoter, the herbicide Liberty™ (DL-phosphinothricin) solution was applied to 2.5-5.0 cm segments of intact leaves of putative transformed and non-transformed plants at 0.1% (v/v) final concentration. Within 3 days, the entire leaf segments of the nontransformed plants and the putative transformed plants that did not contain the *bar* gene developed severe or unevenly partial necrosis. Only Jag#A plant that contained the *bar* gene, showed strong resistance to the herbicide. This evidence indicated the presence of active *bar* gene in Jag#A plant (Fig. 5).

Southern blot analysis for detection of bar and HVA1 gene

To demonstrate the integration of *bar* gene into Jag#A genome by Southern blotting, the genomic DNA from Jag#A and a nontransformed plant was digested with *EcoR*V and probed with ^{32}P-labeled 579 bp *bar* fragment. The fragment used for making labeled probe was obtained by digestion of pAHC20 DNA with restriction enzymes *Bgl*II and *BamH*I. As shown in Fig. 6, the expected 0.87 kb band containing the *bar*

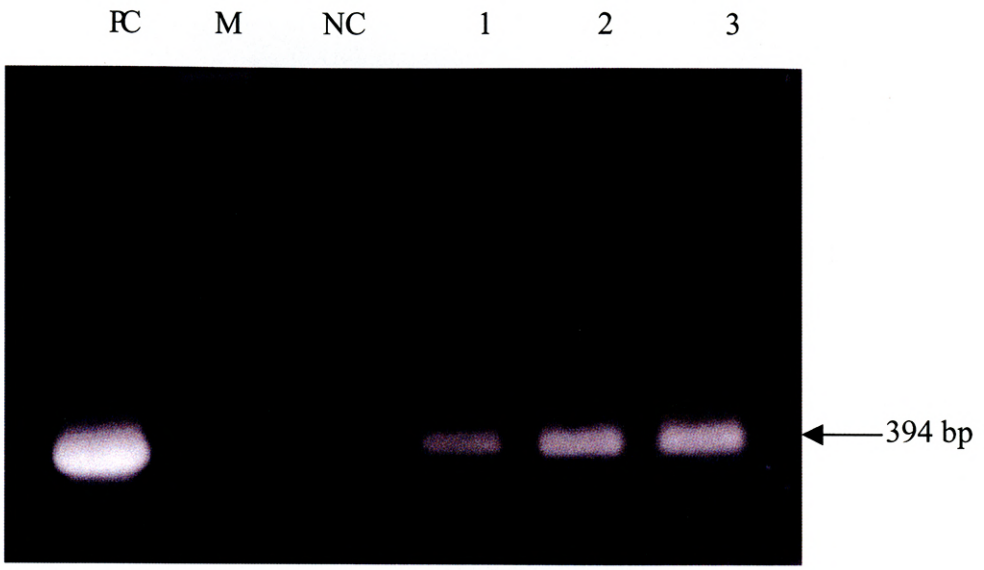

Fig 4. PCR analysis for the *bar* gene from each tiller of Jag#A, transgenic wheat plant

PC : positive control, plasmid DNA, pBy520

NC : negative control, genomic DNA from a nontransformed wheat plant

The numbers indicated the genomic DNA sampled from the leaves of each tiller of Jag#A putative transformed wheat plant

Ten ng of pBy520 was used as template and ~250 ng of genomic DNA was used as template for both negative control and transgenic plant

The expected size of the amplification product is 394 bp, as shown by the arrowhead

Fig 5. Symptoms of wheat leaves after 5 d of 0.1% (v/v) Liberty application

NC1 : A leaf from nontransformed plant derived from seed

NC2 : A leaf from transformed plant that was negative for the *bar* gene

Jag#A : A leaf from putative transformed plant that was positive for the *bar* gene by PCR analysis

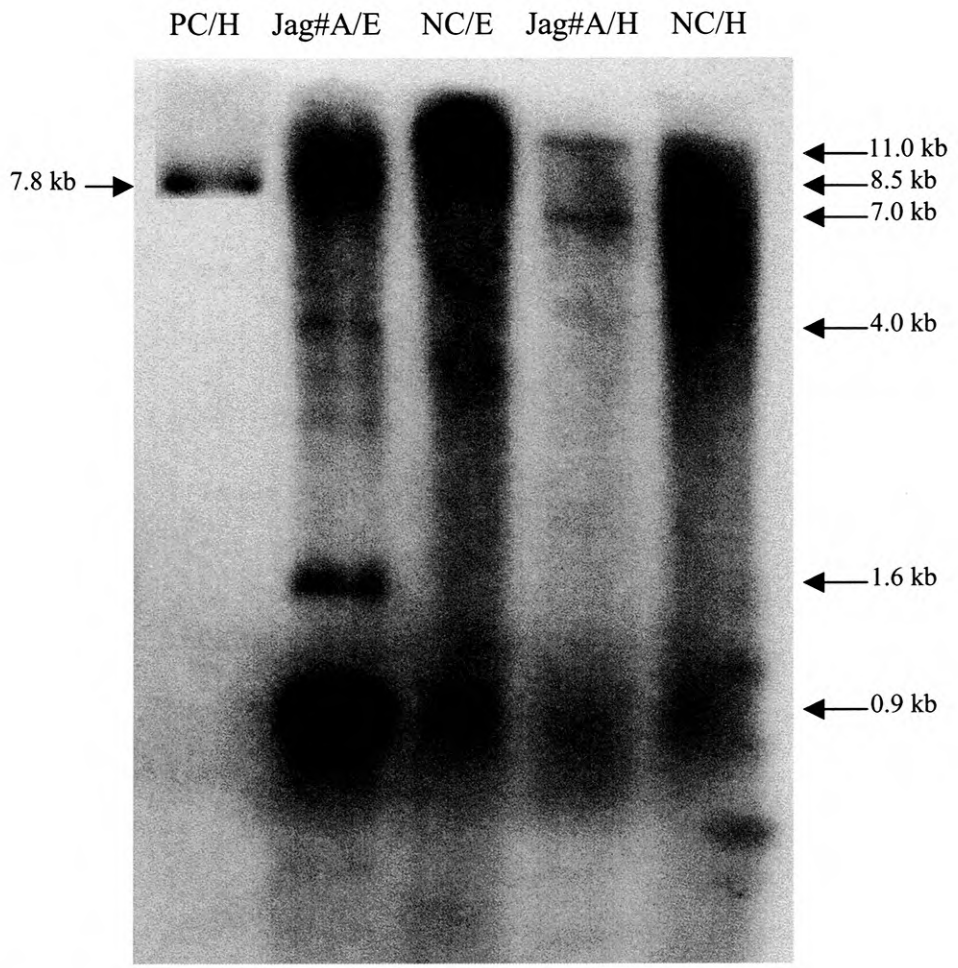

Fig 6. Southern blot analysis for detection of the *bar* gene

PC/H : positive controls; pBy520 was digested with restriction enzyme *Hind*III

NC/E and NC/H : negative controls; genomic DNA from a nontransformed plant which was digested with restriction enzyme *EcoR*V and *Hind*III, respectively

Jag#A/E and Jag#A/H : genomic DNA from Jag#A transgenic plant which was digested with restriction enzyme *EcoR*V and *Hind*III, respectively

^{32}P-labeled 586 bp *bar* fragment was used as a probe

gene and *nos* 3' region was detected in Jag#A plant but was absent in the nontransformed plant. There were also 3 additional higher molecular weight bands observed in the DNA from Jag#A plant.

The genomic DNA from Jag#A and a nontransformed plant were also digested with restriction enzyme *Hind*III and probed with 579 bp *bar* fragment. Two distinct bands were detected in the Jag#A plant while no band was detected in the nontransformed plant (Fig. 6). These results confirmed the results of previous PCR analysis for the presence of *bar* gene and the Liberty™ test for the expression of *bar* gene.

For the detection of *HVA1* gene by Southern blot analysis, genomic DNA was isolated from Jag#A plant, which tested positive for the presence of *bar* gene by PCR and Southern blotting and this plant also was resistant to 0.1% (v/v) herbicide Liberty™. To release the 2.74 kb fragment containing *HVA1* (1 kb) along with *Pin*2 3'-region and, CaMV 35S, the promotor of *bar* gene, restriction enzyme *EcoR*V was also used to digest the genomic DNA from Jag#A plant and from a nontransformed plant, which served as a negative control. The 1 kb *HVA1* probe was released from pBy520 by digestion with the combination of restriction enzymes *Hind*III and *BamH*I. As shown in Fig. 7, the expected 2.74 kb fragment was detected in Jag#A plant while it was absent in the negative control. There also were two distinct bands detected in addition to the 2.74 kb major band in Jag#A plant and they were absent in the negative control. Because the wheat genome contains homologues of the barley *HVA1* gene (Curry et al., 1991), there were additional corresponding bands detected in both transgenic and control plants.

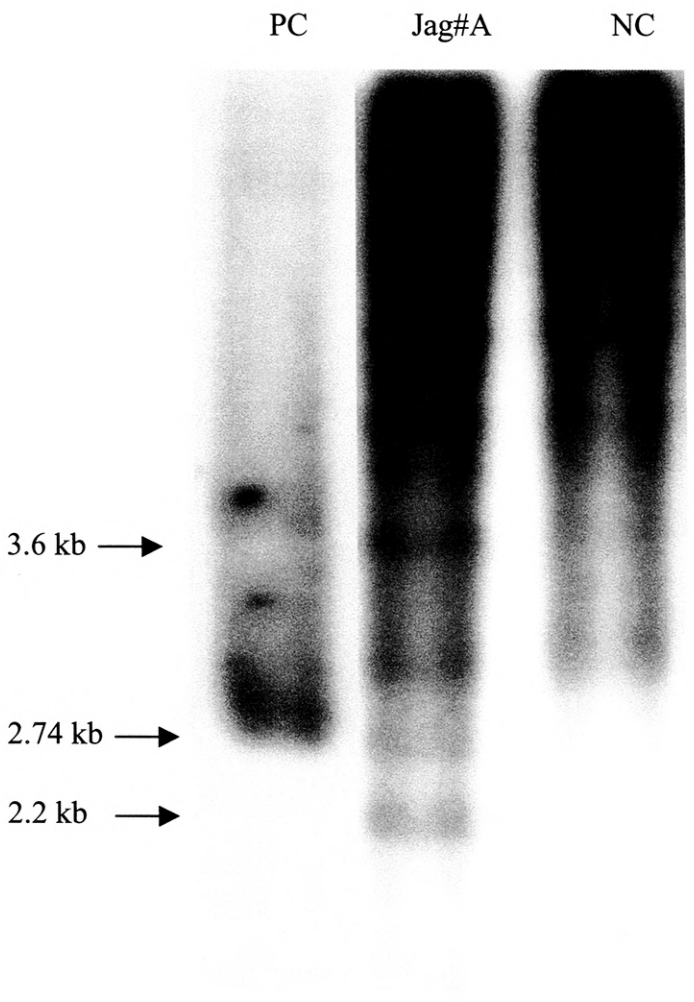

Fig 7. Southern blot analysis for detection of the *HVA1* gene with *EcoR*V restriction digestion

PC : positive control; plasmid DNA, pBy520

NC : negative control; genomic DNA from nontransformed plant

Jag#A : genomic DNA from the putative transgenic plant

^{32}P-labeled 1 kb *HVA1* fragment was used as a probe

Western blot analysis

To examine the expression of the barley *HVA1* gene, Western blot analysis was performed on primary putative transgenic plants. Crude proteins were extracted from the leaves of these plants. In this experiment, the positive control was the protein extract from transgenic rice containing the barley *HVA1* gene and the protein extract from a non-transformed wheat plant derived from a seed was used as a negative control. Protein extracts were subjected to 15% SDS-PAGE and then Western blot analysis. As shown in Fig. 8, an immunoreactive band of 27 kDa *HVA1* protein was detected in Jagger#A plant while it was not detectable in the negative control.

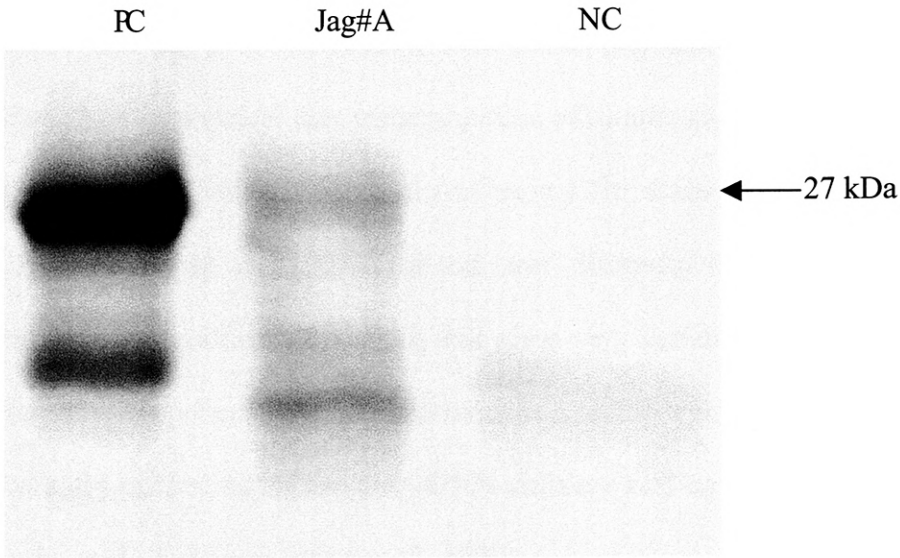

Fig 8. Western blot analysis for detecting expression of barley *HVA1* gene in transgenic wheat plant

PC : positive control, protein extract from a transgenic rice which expressed *HVA1* protein

NC : negative control, protein extract from nontransformed wheat plant

Jag#A : protein extract from a transformed wheat plant cv. Jagger which was obtained via microprojectile bombardment with the *HVA1* gene

DISCUSSION

Transformation technology has been demonstrated in several monocot crops including wheat and the procedures have been developed and optimized by several research groups, but, in practical use, transformation of monocots still has not been very successful (Altpeter et al. 1996; Blechl and Anderson 1996; Barro et al., 1997; Cheng et al., 1997; Dobrzanska et al, 1997; Takumi and Shimada 1997). Microprojectile bombardment, despite significant advances, still gives very variable results (Klein et al., 1992) and regeneration potential of the plant remains a major problem (Perl et al., 1992). Several factors are critical for optimizing DNA delivery and expression (Klein et al., 1988a,b; Kartha et al., 1989 and Wang et al., 1989).

In this study, Jagger (hard red winter wheat) and Lakin (hard white winter wheat) were selected as recipient plants because they have contributed immensely to the economy of the Southern Great Plains. Jagger is one of the leading wheat cultivars in Kansas. According to Kansas Agricultural Statistics, Jagger gained acreage in all districts, accounting for nearly 30% of the State's wheat area. Because they are well-suited to the state's climate and soils, hard white winter wheats, like Lakin and Heyne, also have excellent potential to be succesful crops in Kansas. However, from our experience and others reports, genetic transformation of winter wheat is difficult. According to most reports in wheat transformation, the achievement mostly has been made in spring wheat. Due to the genotype dependence and the optimization of transformation conditions, most elite breeding wheat cultivars still cannot be used for gene transformation. As mentioned in Maës et al. (1996), the relative importance of genotype effect prevailed over all other factors (physical, physiological and chemical

factors). The response of isolated scutella to the induction of somatic embryogenesis showed a genotype-dependent effect, irrespective of medium used. This confirmed that genotype has a major impact on the embryogenic potential of a specific cultivar. In our screening experiments, without bombardment and selection, Jagger and Lakin exhibited good regeneration ability in tissue culture processes compared to Heyne (hard white winter wheat). In subsequent experiments, only immature embryos from Jagger and Lakin were used as donor explants.

From previous transformation experiments (data not shown), when 5 mg/l bialaphos was used as the selective agent and was removed at the second week stage in rooting medium, with approximately 2,000 and 300 bombarded calli from wheat cv. Jagger and Lakin, respectively, no transgenic plant were recovered. A large number of escapes were identified. However, this problem could be overcome in subsequent experiments by replacing the selection agent to 5 mg/l ammonium glufosinate and maintaining selection throughout the rooting stage. There were fewer escapes under selection with 5 mg/l ammonium glufosinate and prolonged selection period than with bialaphos. In fact, even though 10-15% of putative transformed plantlets were obtained during the rooting stage under 5 mg/l ammonium glufosinate, they gradually became necrotic and died later. Only 0-1% of putative transformed plants survived the selection process. For successful microprojectile bombardment, we found that a particle travel distance of 8 cm was more effective than a distance of 10 cm for wheat transformation. With this improvement in our protocol, substantial reduction of labor and time requirements has been achieved. The concentration of 5 mg/l of ammonium glufosinate was sufficiently effective to be used as for selection even though, escapes still occurred.

We also observed variability in the response to the transformation and tissue culture processes of the different batches of donor plants. According to Vasil et al. (1993), the ability of immature embryos to form embryogenic calli varied considerably and depended greatly on the conditions under which the donor plants were grown. They also found that callus sensitivity to the selection agent and its concentration was affected by the nature of callus formation. As the efficiency of transformation differs between the batches of explants used, the comparisons were only made within an experiment involving the same batch of immature embryos (Altpeter et al., 1996).

Some suggestions based on our experiences that may help to improve the transformation efficiency and reduce escapes are : (1) utilize only healthy immature embryos at the optimal stages (13-15 d after anthesis); (2) allow regenerating shoots to grow up to at least ~0.8-1.0 cm in length, prior to transferring to rooting medium in order to prevent overgrowth of roots without shoot extension; (3) subcultures should be made bi-weekly to maintain abundant nutrient supply and the effectiveness of the selection agent; (4) discard those calli that remain shootless one month after being kept in the regeneration medium; (5) transfer only healthy plantlets with a strong root system to the soil; and (6) for winter wheat cultivars, vernalization is needed.

For the molecular analyses of the transgenic wheat plants, PCR analysis was the fastest and most sensitive screening method to detect the presence of *bar* gene. It was carried out at the first step due to the simplicity of the technique. Less leaf tissue is required and the technique is less time-consuming. All the tillers from each putative transformed plants were analyzed to ensure that this transgenic plant was not chimeric.

The transgenic Jagger wheat plant obtained in our experiment was finally proved to be uniformly transformed because all three tillers were positive in PCR.

From Southern blot analysis of the transgenic plant DNA, it was shown that a 2.74 kb hybridization band (*EcoR*V digested) including the intact 1 kb *HVA1* coding region along with the *Pin*2 3'-region and the CaMV 35S promoter of *bar* gene, was integrated into the genome of Jagger wheat plant, which constitutively expressed the *HVA1* protein. However, this transgenic plant also contained 2 extra hybridization bands of varying sizes, which indicated that there were at least 3 loci of transgene integration. Multiple copies of the intact *bar* gene also were detected in this transgenic plant, although there were at least 4 different bands carrying the *bar* gene as revealed by the *EcoR*V restriction digestion. The *bar* gene was shown to be functional by the 0.1% Liberty painting.

By Western blot analysis, the corresponding 27 kDa *HVA1* protein was detected in our transgenic wheat plant while it was absent in the nontransformed wheat plant. However, when the same amount of protein was loaded, the protein expression level in the transgenic wheat was much less than the level in the transgenic rice. This is probably due to the promoter. In this experiment, the barley *HVA1* gene was driven by the rice *Act1* promoter. Therefore it is possible that this promoter works more efficiently in rice tissue than in wheat.

In summary of this study, (1) a transgenic wheat plant has been obtained using microprojectile bombardment with 0.5% transformation efficiency; (2) the microprojectile bombardment is a reliable method for wheat transformation; (3) it is feasible to transfer genes from different species like barley to a wheat genome and

expressing functional protein could be achieved in the transformants; (4) the rice *Act1* promoter has proved to be funtional in wheat cv. Jagger; and (5) the microprojectile bombardment results in multiple-copies of gene integration.

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