

Research Article

Genetic Transformation of Common Bean (*Phaseolus vulgaris* L.) with the *Gus* Color Marker, the *Bar* Herbicide Resistance, and the Barley (*Hordeum vulgare*) *HVA1* Drought Tolerance Genes

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Five common bean (*Phaseolus vulgaris* L.) varieties including “Condor,” “Matterhorn,” “Sedona,” “Olathe,” and “Montcalm” were genetically transformed via the Biolistic bombardment of the apical shoot meristem primordium. Transgenes included *gus* color marker which visually confirmed transgenic events, the *bar* herbicide resistance selectable marker used for *in vitro* selection of transgenic cultures and which confirmed Liberty herbicide resistant plants, and the barley (*Hordeum vulgare*) late embryogenesis abundant protein (*HVA1*) which conferred drought tolerance with a corresponding increase in root length of transgenic plants. Research presented here might assist in production of better *P. vulgaris* germplasm.

1. Introduction

The common bean (*Phaseolus vulgaris* L.) is a very important source of vegetable protein, especially in those regions of the world in which animal proteins are scarce. Common bean provides 22% of the total protein requirement worldwide [1]. Conventional breeding has contributed significantly to the trait improvement of *P. vulgaris*. However, breeding cannot add certain genes that do not exist naturally in the *P. vulgaris* gene pool. Due to this limitation of plant breeding, new trait improvement approaches such as interspecific horizontal gene transfer via genetic engineering need to be utilized in order to complement the limitations encountered by conventional breeding of this crop [2, 3].

Mostly, *Agrobacterium*-mediated transformation and the gene gun microprojectiles bombardment method have been used for genetic transformation of *P. vulgaris*. However, neither system has shown as high as those seen in genetic transformation of cereals [4]. Researchers have unsuccessfully attempted to transform *P. vulgaris* protoplast, either via polyethylene glycol or electroporation [5]. A relatively advanced *Agrobacterium*-mediated transformation of *P. vulgaris* has been reported on the use of sonication and

vacuum infiltration for transfer of a group of 3 LEA (late embryogenesis abundant protein) genes from *Brassica napus* [6]. Although the transformation efficiency using this system was low, transgenic plants exhibited a high growth rate under salt and water stress. A recent report [7] on transformation of *P. vulgaris* varieties Mwitmania and Rose coco using the *gus* color marker gene reveals the importance of specificity of *Agrobacterium* strains in expression of *gus* gene in *P. vulgaris*. For example, infecting of *P. vulgaris* explants with EHA 105 (pCAMBIA 1201) or EHA 105 (pCAMBIA 1301) resulted in blue GUS coloration; however, it did not show the GUS expression when the explants were infected with LBA 4404 (pBI 121) *Agrobacterium* strain.

Using Biolistic bombardment of a construct containing the *bar* gene, Aragão et al. [3] developed transgenic *P. vulgaris* which conferred resistance to glufosinate ammonium, the active ingredient of Liberty herbicide (Aventis, Strasbourg, France), at concentrations of 500 g ha⁻¹ in greenhouses and 400 g ha⁻¹ in the field. *P. vulgaris* was also genetically engineered by Bonfim et al. [8] using RNAi-hairpin construct to silence the AC1 region of the viral genome of Bean Golden Mosaic Gemini Virus (BGMGV). However, out of 2,706 plants, only 18 putative transgenic lines were obtained.

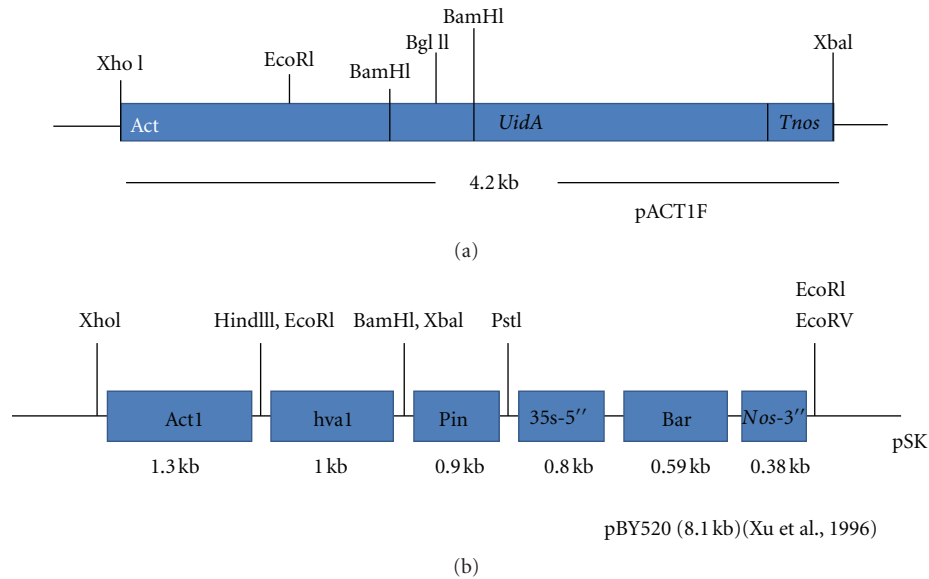


FIGURE 1: Plasmid constructs. (a) Linear map of pACT1F plasmid vector. Rice actin promoter (Act1), *gus* gene (*uidA*), and nopaline synthase terminator (*Tnos*); (b) linear map of pBY520 plasmid vector. Rice actin promoter (Act1) and potato protease inhibitor II (Pin II-3') terminator, Barley or *Hordeum vulgare* (*HVA1*) LEA 3 gene, Cauliflower Mosaic Virus 35S promoter, *bar* gene and nopaline synthase terminator (*Nos-3'*).

Of the 18 putative transgenic plants, only one plant exhibited resistance to the virus. Field trials of the progenies of the single transgenic plant showed resistance to this virus [9]. Vianna et al. [10] developed an approach of transferring the transgene assembly as fragment pieces of DNA, as opposed to the entire plasmid into *P. vulgaris*. A protocol was published [11] on a relatively efficient genetic transformation of *P. vulgaris*. Due to the “troublesome” nature of *P. vulgaris* genetic transformation, an article describes a method called “transgenic composite” of *P. vulgaris* via the use of *Agrobacterium rhizogenes* transformation of derooted seedlings [12].

The efficiency of genetic engineering of *P. vulgaris* has remained a challenge. A relatively recent report explains the effect of *in vitro* conditions on indirect organogenesis (multiple shoots from meristem and cotyledon-derived callus regeneration) for production of an average of 0.5 shoot per callus clump. Indirect regeneration of different genotypes of *P. vulgaris* was also reported [13]. Kwapata et al. [14] cite that an *in vitro* culture of a single apical shoot meristem primordium could produce as many as 20 multiple shoots, which is a relatively higher number as compared to the work previously presented. However, this *in vitro* regeneration efficiency is still very low when compared to the desired 100s regenerated from the *in vitro* cultures of each apical shoot meristem primordia of cereal crops [4].

Genetic transformation of *P. vulgaris* can improve the biotic and abiotic stress tolerance. Biotic stress factors such as diseases result in *P. vulgaris* yield loss. Brazil just announced [15] the commercial use of golden mosaic virus resistant *P. vulgaris* that was developed via RNA interference by blocking the replication of the virus gene [8]. This is indeed a major step in the acceptance of biosafety of transgenic *P. vulgaris*.

Also, researchers from Denmark recently reported cloning of the bean common mosaic virus (BCMV) gene and its application for development of BCMV resistance [16].

Biotic stresses, including drought cause plants to lose cellular turgidity, followed by the aggregation and misfolding of proteins and yield losses [17]. A major group of abiotic stress tolerance genes coding for the late embryogenesis proteins include a class of heat shock proteins (Hsp) that are extremely hydrophilic and resilient towards heat, such that they do not coagulate at boiling temperatures. The LEA proteins play a role in water binding, ion sequestration, and macromolecule and membrane stabilization [18]. In the research presented here, the barley *HVA1* [19] gene was transferred into *P. vulgaris*, as this gene encodes a type III LEA protein. The Barley *HVA1* gene has previously been transferred to rice [20], wheat [21, 22], sugarcane [23], creeping bentgrass [24], mulberry [25], and oat [26, 27]. In all cases, plants developed tolerance to abiotic stresses such as drought and/or salt. Here we report the transfer of Barley *HVA1* gene to different varieties of *P. vulgaris* and report the development of drought tolerance of transgenic plants at greenhouse level.

2. Materials and Methods

2.1. Plasmids and Explant. Two different plasmid vectors were used in this research (Figure 1). Plasmids used included (a) pACT1F harboring the *gus* gene and (b) pBY520 harboring the *HVA1* and the *bar* gene, which confers drought tolerance and Liberty herbicide (glufosinate ammonium) resistance, respectively.

Explant preparation: the explant used to standardize the genetic transformation was *P. vulgaris* var. “Sedona.”

Dry seeds were rinsed in tap water for 1 min, then rinsed three times with distilled water, soaked in 75% ethanol for 4 min, and again rinsed three times with distilled water. Then, the seeds were soaked in 20% commercial Clorox while steering for 15 min.

Seed coats of the surface-sterilized seeds were removed, and meristems were dissected under a light microscope under a laminar flow hood. The meristem dissection took place by removal of the cotyledons and the hypocotyls, leaving the meristem as an intact explant.

The meristem explants were cultured in Murashige and Skoog (MS) [28] medium containing 2.5 mg^{-1} benzyl adenine (BA; Sigma-Aldrich, Inc. Steinheim, Germany) and 0.1 mg^{-1} indole acetic acid (IAA; Sigma-Aldrich, Inc. Steinheim, Germany). Cultures were maintained under *in vitro* conditions and in a dark chamber for 5–7 days or until the explants grew to about 5–7 mm long. Then, 10 of the elongated apical meristems were placed in a circle in a Petri dish on top of MS medium, bombarded with gene constructs using the Biolistic gene via the helium particle delivery model PDS-1000 (DuPont, Wilmington, DE).

The pACT1-F construct containing the *gus* gene was coated onto $50 \mu\text{g L}^{-1}$ of $10 \mu\text{m}$ tungsten particles with 2.5 M calcium chloride and 0.1 M spermidine suspended in a solution of 1:1 (v/v) of 75% ethanol and 50% glycerol. The coated plasmid DNA was bombarded into the explants using three levels of pressure (500, 1000, or 1100 psi), plasmid concentrations of $1.5 \mu\text{g}$ or $3.0 \mu\text{g}$, and with three levels of bombardment frequencies (1, 2 or 3 time). A total of 10 apical meristems were used for each bombardment condition.

The bombarded shoot meristems were transferred to regeneration medium [14] and kept under *in vitro* condition at room temperature with 16 h photoperiod and light intensity of $45\text{--}70 \mu\text{mol m}^{-2}\text{s}^{-1}$.

The bombarded shoot meristems were histologically stained to visualize the *gus* gene expression, and three longitudinal hand-cross-sections of each bombarded shoot meristem were made to identify the bombardment criteria that lead to expression of *gus* gene in relative location of *P. vulgaris* meristem subepidermal layer. Mean of transient transformation efficiencies (number of meristems showing blue spots) was used as preliminary data to identify the most acceptable criteria of bombardment (Table 1).

The most effective criteria were then used for stable transformation of the five varieties of *P. vulgaris*. The GUS histological assay bombarded versus control wild-type meristems included histochemical staining with 5-bromo-4-chloro-3-indoyl- β -D-glucuronic acid salt (X-gluc). Samples were dipped into GUS substrate buffer, according to published records [29], and incubated at 37°C for 24 hours. The tissue samples were washed with 100 percent ethanol to remove other colorations.

The statistical design used in this portion of research was a completely randomized design (CRD). An Analysis of Variance (ANOVA) was used to test the statistical significance at an alpha level of 0.001. Standard deviations were used to compare variability.

2.2. Stable Genetic Transformation. Stable genetic transformation of *P. vulgaris* was performed using the Biolistic delivery for bombardment of a 1:1 ratio mixture of the two plasmids into the apical shoot subepidermal cell layer area using the ideal bombardment criteria (Table 1). The bombarded explants were cultured in regeneration media [14] without the use of any chemical selections for 24 hours.

The selection of stable transgenic plants was based on the use of *gus* color marker gene and 4 mg L^{-1} of glufosinate ammonium selection for the *bar* herbicide resistance marker gene. The *in vitro* regeneration of putatively transgenic *P. vulgaris* explants followed a previous report [14].

2.3. Confirmation of Transgene Integration and Expression

2.3.1. Polymerase Chain Reaction (PCR). Polymerase chain reaction (PCR) was used for detection of integration of *bar* and *HVA1* transgenes in four generations ($T_0\text{--}T_3$) of plants that were putatively transformed with Biolistic gun. The primers used were (1) *bar* F, 5'-ATG AGC CCA GAA CGA CG-3' (forward primer); *bar* R, 5'-TCA CCT CCA ACC AGA ACC AG-3' (reverse primer); (2) *HVA1* F, 5'-TGG CCT CCA ACC AGA ACC AG-3' (forward primer); *HVA1* R, 5'-ACG ACT AAA GGA ACG GAA AT-3' (reverse primer).

2.3.2. Southern Blot Hybridization. The Southern blot hybridization analysis was conducted to determine the stability of transformation and to determine the copy numbers of the *bar* and *HVA1* transgenes. The DIG High Prime DNA Labeling and Detection Starter Kit (Roche Co., Cat. No. 1 585 614) was used as per manufacturer's instructions. Transgenic and control wild-type nontransgenic genomic DNA was isolated using methods described [30]. The DIG-labeled probes for *bar* and *HVA1* were synthesized using primers for specific genes as described previously. Those transgenic plants that integrated 1-2 copies of transgenes were kept for further studies.

2.3.3. Northern Blot Hybridization. Northern blot analysis was conducted using the DIG-labeled Northern Starter Kit (Roche Co., Cat. No. 12039672910). Total RNA from the leaves of transgenic and the control wild-type nontransgenic plants was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO) as per manufacturer's instructions. A total of $30 \mu\text{g}$ of RNA per sample was loaded onto a 1.2% (m/v) agarose-formaldehyde denaturing gel as described [31] and transferred to a Hybond-N+ membrane (Amersham-Pharmacia Biotech) and fixed with a UV crosslinker (Stratalinker UV Crosslinker 1800, Stratagene, CA). The RNA or DNA DIG-labeled probe, containing the coding region of the gene of interest, was used for detection of transcripts.

2.4. Biological Activity Tests

2.4.1. Herbicide Resistance Assay. Following a glufosinate ammonium *in vitro* culture kill curve studies (data not shown), an optimum 4 mg L^{-1} of glufosinate ammonium

was used in the *in vitro* culture of putatively transgenic shoot regeneration and rooting media.

Different concentrations of Liberty herbicide (50, 100, 150, 250, or 350 mg L⁻¹) were used to find the ideal foliar spray concentration of trifoliolate transgenic plants.

In vitro germination of progeny seeds in MS medium [28] containing 4 mg L⁻¹ of glufosinate ammonium was used to identify segregation ratio of the *bar* transgene in transgenic progenies.

2.4.2. Drought Tolerance Test. The *HVA1* transgenic and wild-type control seeds were collected, and seedlings were grown in 15 cm clay pots containing BACCTO High Porosity Professional Planting Mix (Michigan Peat Company, Houston, TX) in a growth chamber for three weeks or until trifoliolate leaves appeared. Plants were watered daily for 21 days, after which moisture was withheld for 21 days. Then, water was applied to plants continuously for up to 14 days, and the percentage of plants recovered was recorded. Also, percent plant leaf abscission was used as an indirect measure of degree of plant wilting. In reality, the number of green leaves on plants after 21 days of moisture withdrawal was used to find percent plant leaf abscission.

3. Results and Discussions

3.1. Explant. Our results show that the apical shoot meristem primordium might be a good explant for genetic transformation of common beans. The apical shoot meristem in *P. vulgaris* is an undifferentiated meristematic tissue in a small and relatively round shape, which is composed of different cell layers. The top layer or the “Epidermal Cell Layer” divides horizontally and will not differentiate. The layer beneath the Epidermal Cell layer is the “Subepidermal Cell Layer” (also called the primordial cell layer or stem cell layer) normally divides indefinitely and differentiates into gametes resulting into fertile plants. Therefore, it is the Subepidermal Cell Layer that needs to be targeted via the Biolistic gun for genetic transformation. Using the *gus* color marker gene, the researchers of this report tried to standardize the Biolistic delivery bombardment to hit this layer.

3.2. Transient Expression of the *Gus* Marker Gene. Bombarding the explants twice at the approximate distance of 4 cm between the gun barrel and target explants, using a pressure setting of 1100 psi, with a concentration of 1.5 µg of plasmid DNA per bombardment yielded the highest GUS activity efficiency of 8.4% (Table 1). Mean transient transformation was calculated by counting the mean of number of bombarded meristems that showed blue spots.

The transient transformation frequency of the GUS expression is shown in Figure 2. The number of clear blue spots was seen 15 days after bombardment.

3.3. Stable Transformation. PCR was performed for all *bar* and *HVA1* transgenes used, among which results are only shown for integration of *HVA1* transgene in all four *P. vulgaris* cultivars (Figure 3(a)). Southern blot hybridizations were performed in multiple samples of PCR-positive plants,

TABLE 1: Transient expression of GUS using different gene gun pressure (psi), DNA plasmid concentration and bombardment frequency for optimizing the Biolistic bombardment conditions for the pCATIF containing the *gus* gene.

Bombardment pressure (psi)	Concentration of plasmid DNA (µg)	Bombardment frequency	Mean transformation percent
500	1.5	1	0.1 ± 0.04
500	1.5	2	0.2 ± 0.10
500	1.5	3	0.4 ± 0.30
500	3	1	0.1 ± 0.04
500	3	2	0.6 ± 0.32
500	3	3	0.7 ± 0.32
1000	1.5	1	2.9 ± 0.67
1000	1.5	2	3.9 ± 1.4
1000	1.5	3	5.1 ± 1.2
1000	3	1	5.6 ± 1.0
1000	3	2	8.1 ± 0.3
1000	3	3	7.4 ± 1.0
1100	1.5	1	7.2 ± 0.70
1100	1.5	2	8.4 ± 0.74
1100	1.5	3	8.2 ± 0.50
1100	3	1	7.5 ± 0.69
1100	3	2	4.8 ± 0.93
1100	3	3	3.3 ± 0.92

among which data are only shown for integration of *HVA1* gene in different *P. vulgaris* cultivars. After Southern blot hybridization analysis, transgenic plants that showed the integration of at most two copies of transgenes (e.g., see Figure 3(b)) were kept for transcription analysis. Transcription analysis via RT-PCR showed that *HVA1* has transcribed in all transgenic plants. However RNA blotting confirmed that only certain transgenic plants sufficiently transcribed their transgenes (e.g., see Figure 3(c)). This is because RT-PCR is much more sensitive than the RNA blotting.

The GUS bioassay was a method of selecting the transgenic shootlets. All Southern blot-positive progenies of *P. vulgaris* varieties (“Matterhorn,” “Condor,” “Sedona,” “Olathe,” and “Montcalm”) showed GUS expression. Figure 4 represents expression of GUS protein in seeds and pods of T3 of “Matterhorn.”

Because glufosinate ammonium was included in the *in vitro* cultures of all putatively transgenic shoots, roots and plantlets, all transgenic plant progenies were resistant to 150 mg L⁻¹ of Liberty herbicide (Figure 5). Lower concentrations did not kill wild-type control nontransgenic plants, and higher concentrations killed transgenic plants as well as their wild-type control non-transgenic counterparts.

Most drought tolerant *HVA1* transgenic plants were “Sedona” and “Matterhorn” which persisted for 21 days without irrigation. They showed symptoms of drought stress but recovered only after three days when moisture application resumed. The wild-type control plants died or showed severe symptoms of drought stress, with most of their leaves

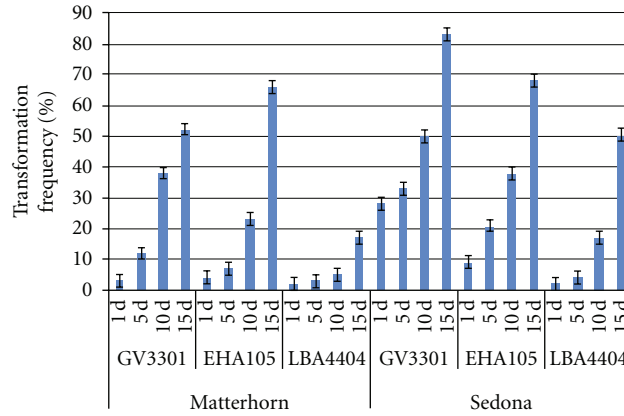


FIGURE 2: Percent transient expression of GUS at different number of days after bombardment.

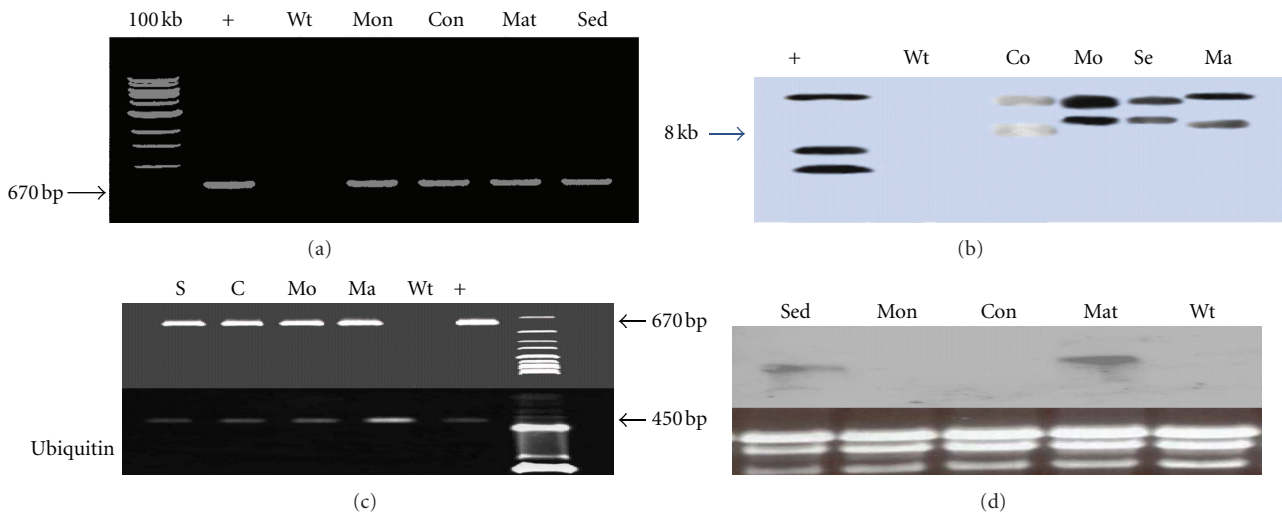


FIGURE 3: Molecular analysis confirming the integration and transcription of *HVA1* transgene in plants. (a) PCR results of T3 transgenic plants. The expected band size is 670 bp; (b) Southern blot hybridization showing integration of *HVA1* gene digested with BamH1. The results indicate that there are two copies of transgene in all varieties. Like in PCR, here the expected band size is 670 bp for *HVA1*. Below the RT-PCR is the cDNA loading control showing the expression of ubiquitin with the expected band size of 450 bp. (d) Northern blot analysis also confirmed the transcription of *HVA1* gene in Sedona and Matterhorn. Wt: wild type shows no transgene integration; C: RNA transcription analysis of *HVA1* gene in T3 transgenic plants. Mat: “Matterhorn” and Sed: “Sedona” showed some expression. The remaining lanes, Wt: wild type, Mon: “Montcalm” and Con: “Condor” showed no transcriptions.

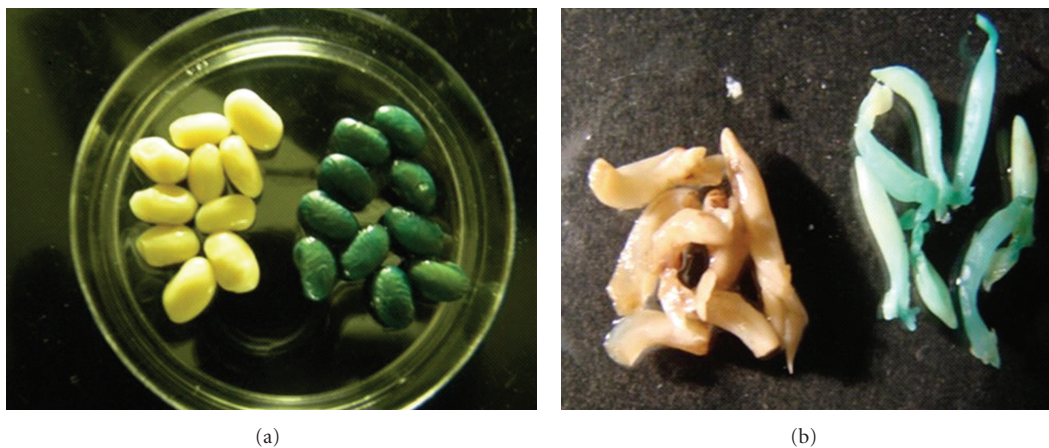


FIGURE 4: GUS biological activity shown after histochemical assays in pods and seeds of T3 “Matterhorn.”

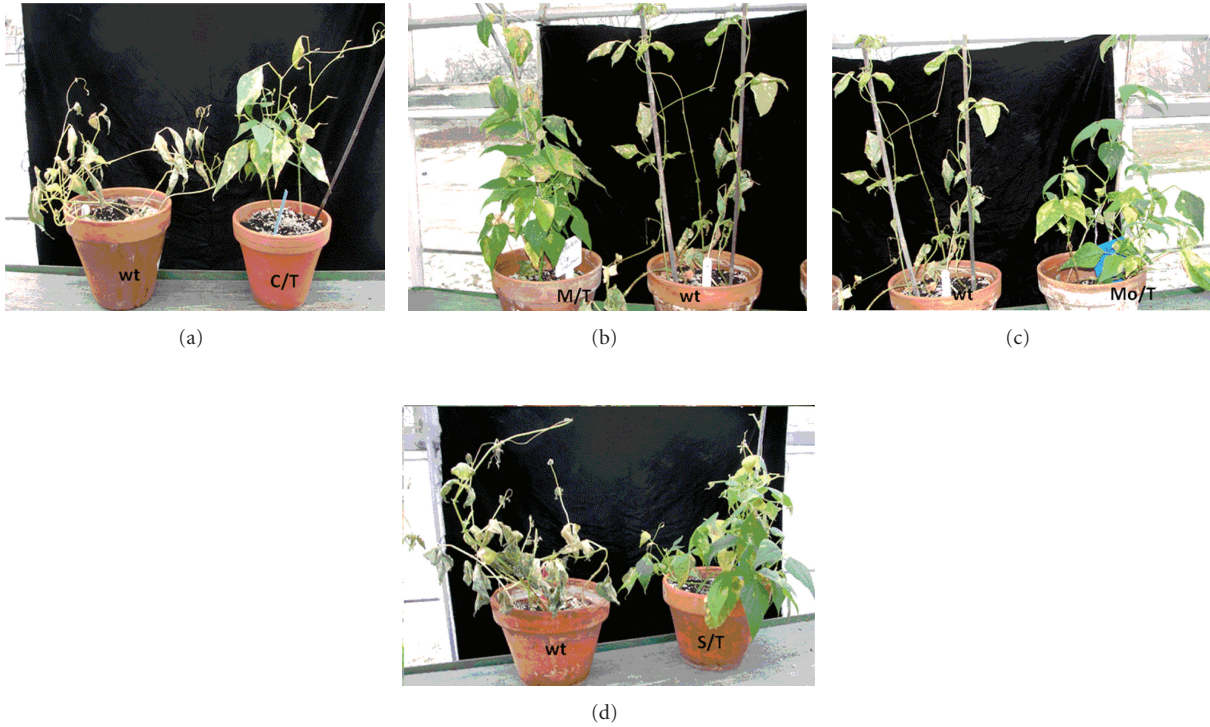


FIGURE 5: Although not completely resistant, the trifoliate stage of the third generation transgenic (T2) plants that had transcribed *bar* gene showed more resistance to foliar spray of 150 mg L^{-1} Liberty herbicide than their wild-type control non-transgenic counterpart plants. “Condor” (a), “Matterhorn” (b), “Montcalm” (c), and “Sedona” (d). “Matterhorn” seems to be more resistant to the herbicide.

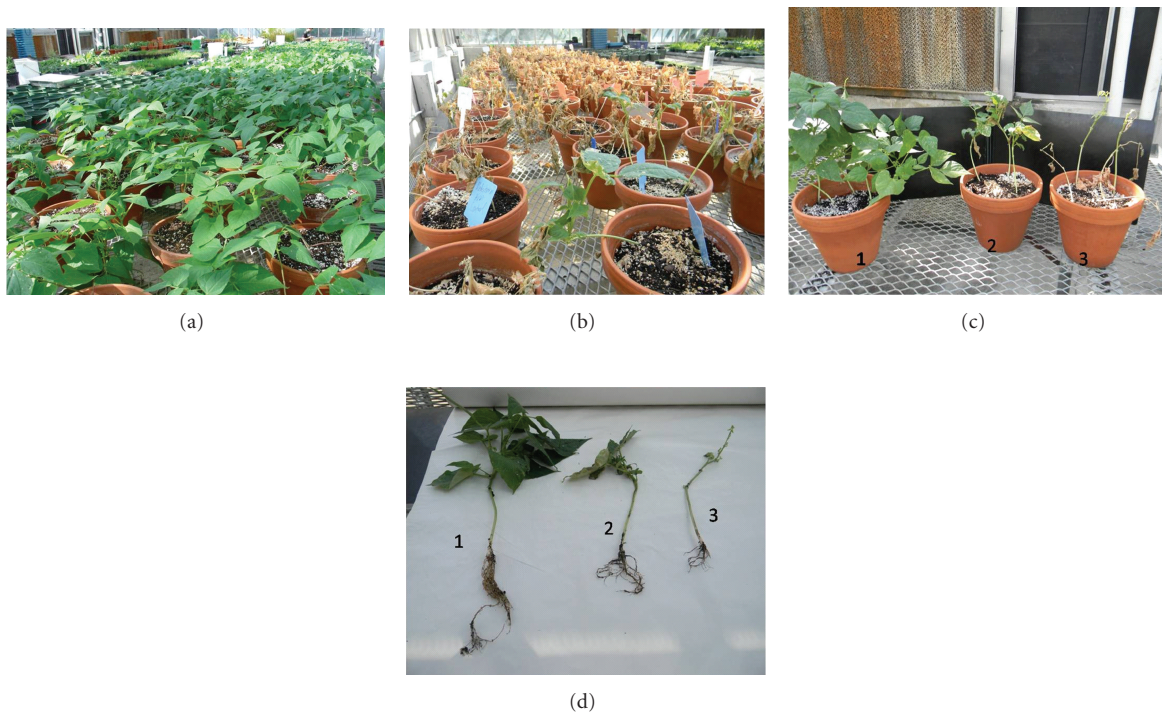


FIGURE 6: Drought tolerance assays. (a) “Matterhorn” plants before drought induction; (b) after 21 days of continuous water withholding; (c) “Matterhorn” drought recovered plants after water reapplication; 1: control non-transgenic plant that was watered throughout the experiment; 2: “Matterhorn” transgenic plant after 21 days of no-irrigation, 3: wild-type non-transgenic plant after 21 days of no-irrigation; (d) root growth in plants after 21 days of drought stress. 1: Control non-transgenic plant roots; these were watered daily, 2: transgenic plant roots after 21 days of no-irrigation, and 3: wild-type non-transgenic plant roots after 21 days of no-irrigation.

being wilted and dehisced (e.g., see Figure 6(b)). The survival rate of control wild-type non-transgenic “Sedona” plants after 21 days of drought was 13.3% and for its HVA1 transgenic plants of the same variety was 33.3%. In case of “Matterhorn,” the survival rate of control wild-type non-transgenic plants was 20%, and its transgenic counterpart of the same variety was 53.3%. Withdrawal of irrigation for more than 21 days resulted in the death of both control wild-type non-transgenic and HVA1 transgenic plants of “Sedona” and “Matterhorn” varieties.

The percent leaf wilting of transgenic “Sedona” plants was 78% and for its wild type was 91%. In the case of “Matterhorn,” percent leaf wilting of transgenic plants of “Matterhorn” variety was 72% as compared to the wild-type control non-transgenic plants which were 88%.

Over all, the root growth of HVA1 transgenic plants with least percent wilting was more robust than wild-type plants under stress, but less developed than wild type plants under a normal moisture regime (e.g., see Figure 6(c)).

In a preliminary experiment, the average root length measurement after 21 days of water withdraw for “Sedona” HVA1 transgenic plants was 15 cm and for wild-type plants was 11 cm. For “Matterhorn” variety was 72% as compared to the wild-type control non-transgenic plants which was 88%. In contrast, for control wild type plants under normal irrigation without water withhold, the average root length was 28 cm.

The researchers of this paper exposed transgenic plants transcribing the HVA1 gene to drought prior to testing of plants for drought tolerance. The promoter deriving the HVA1 in this work is rice actin 1 promoter which is known to be a constitutive promoter. Transgenic plants might have shown more drought tolerance should the promoter used was an inducible one, such as *Arabidopsis* rd29 promoter [32].

4. Conclusions

GUS assay was essential to identify the relative location of the subepidermal area of explants as the target for Biolistic bombardment.

All plants transformed with the *bar* Liberty herbicide resistance gene showed stable expression of this gene because of continuous *in vitro* culture selections of explants, shootlets, and plantlets in media containing the active ingredient of this herbicide.

Our studies of transgenic *P. vulgaris* that expresses barley HVA1 transgene agree with an earlier report [33] in which “Matterhorn” possesses a genotypic advantage over “Sedona” in terms of naturally tolerating drought.

The expression of barley HVA1 gene in *P. vulgaris* resulting in drought tolerance agrees with results obtained from transfer of this gene into other crops and their tolerance to drought and/or salt [20–27].

Further studies are needed to locate the precise location of the subepidermal cell layer, possibly via the use of GUS monoclonal antibody followed by laser microscopy because GUS color easily diffuses from cell to cell.

Further studies are also needed to test HVA1 transgenic *P. vulgaris* at the field level. The research presented here and the genes transferred into common bean varieties might improve the yield and economy of this important crop.

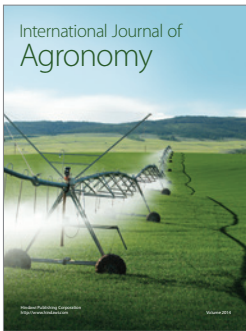
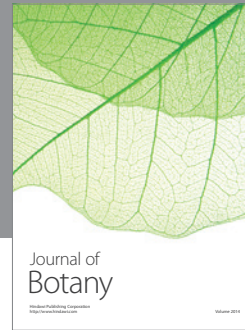
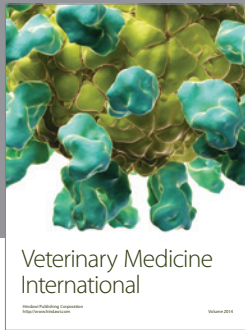
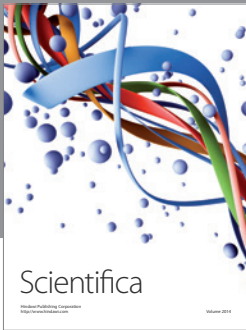
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