通过基因枪和农杆菌介导用 *BADH* 基因转化小麦

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摘要: 土壤盐碱是一种严重障碍作物生产的环境因子, 甜菜碱醛脱氢酶 *BADH* 基因是一种重要的可赋予植物渗透调节抗性的基因。本研究用基因枪法及农杆菌介导法向小麦幼胚和成熟胚愈伤组织导入了 *BADH* 基因。用 PDS-1000/HE 基因枪轰击 2 933 块幼胚愈伤组织, 分化出了 45 株再生植株, 分化率为 1.53%。PCR 分析表明, 其中的 5 株为 *BADH* 基因转化植株。用 PPT 涂抹其叶片, 进一步证实了 PCR 的结果。以小麦成熟胚愈伤组织为受体, 用农杆菌介导转化 1 968 块愈伤, 仅再生出了 5 株绿苗, PCR 检测结果均为阴性。但对其转化愈伤组织的 PCR 检测表明, 外源基因已在受体细胞中实现了整合。以幼胚愈伤组织为受体, 用农杆菌介导转化 2 933 块愈伤, 共再生出了 21 株绿苗。对其进行 PCR 检测, 仅有 5 株为 *BADH* 基因转化植株。转化处理过的幼胚愈伤组织的绿苗再生率(0.72%)高于成熟胚愈伤(0.25%)。与对照相比, 所有的转化植株均能够在 0.5% NaCl (w/w) 条件下正常生长, 表明外源 *BADH* 基因已经整合并表达。

关键词: 遗传转化; 小麦; 农杆菌; 基因枪; *BADH* 基因

中图分类号: S512.01 文献标识码: A 文章编号: 1000-7091(2005)05-0001-09

Transformation of Wheat with *BADH* Through Gene Gun and *Agrobacterium*

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Abstract: Salinity is very severe environmental problem hampering crop production. *BADH* is an important gene for conferring osmotic stress tolerance to plants. In this study gene gun and *Agrobacterium* were employed for the delivery of *BADH* into callus of wheat. Bombardment of 2 933 calli derived from immature embryos resulted in regeneration of 45 plant-lets after selection on phosphinothricin (PPT) with net regeneration rate of 1.53%. PCR analysis confirmed the presence of *BADH* gene in five plants. Results of PPT-leaf painting conformed to PCR test. In case of *Agrobacterium* mediated transformation five plant-lets regenerated from inoculation of 1 968 calli derived from mature embryos but none was PCR positive. PCR test of calli from mature embryos exhibited the successful delivery and integration of transgene. Inoculation of 2 933 calli from immature embryos resulted in regeneration of 21 plant-lets. PCR evaluation of regenerated plant-lets indicated the presence of *BADH* in 5 plants only. Net regeneration rate for immature embryos (0.72%) was higher than that of mature embryos (0.25%). Transformed plants obtained through *Agrobacterium* and gene gun were able to thrive in 0.5% NaCl (w/w) without stunted growth and wilting as compared with check plants indicating the successful integration and expression of *BADH*.

Key words: Transformation; Wheat; *Agrobacterium*; Gene gun; *BADH*

收稿日期: 2005-08-08
基金项目: 河北省自然基金项目(303606)
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1 Introduction

A significant progress is being made in the genetic improvement of wheat through genetic engineering but at a slow pace. Since the production of first fertile transgenic wheat plants several attempts have been made to transform different varieties of wheat using genes for various traits of common interest. Expression of low molecular weight glutenin subunits in the starchy endosperm[1], acceleration of flowering time by more than a month by insertion of RNA-interference genes[2], transgenic wheat for drought and salt tolerance[3,4] are recent advances in genetic improvement of wheat.

Salinity is very important environmental stress hampering crop production. It is an ever-present threat to crop yield especially in countries like China and Pakistan where irrigation is an essential aid to agriculture. Breeding crops for stressful environment is one way for increasing productivity in the areas subjected to abiotic stresses. However, traditional approaches at transferring resistance to crop plants against abiotic stresses are limited by genetic and physiological complexity of stress tolerance traits. Several genes control tolerance to stress and their simultaneous selection is difficult. Some undesirable genes that are incorporated during cross breeding require a tremendous effort to eliminate these genes. Additionally, selection for stress tolerance under field conditions is also difficult due to lack of efficient procedures. So the attempts to improve salt tolerance of crops through conventional breeding have met with limited success. Genetic engineering offers an alternative approach for developing stress tolerant crops. It is faster and more precise means of achieving improved tolerance. It avoids incorporation of unwanted genes. The genetic engineering coupled with traditional plant breeding efforts seems to be the most suitable strategy to exploit the deteriorating land resource for increased food production to cope with the growing food demands of burgeoning world population. It offers a convenient and rapid approach for improvement of stress tolerance. Some progress has been made in improvement of wheat for abiotic stress resistance through genetic transformation.

Salt tolerance requires accumulation of solutes to function in osmotic adjustment and osmoprotection. Glycine betaine is one of the important osmoprotectants involved in osmoregulation. It preserves thylakoids and plasma membrane integrity after exposure to saline conditions. In general, accumulation of significant quantities of betaine has been noted in plants that tolerate salinity.

Higher plants synthesize glycine betaine from choline in a two-step reaction that is catalyzed by two enzymes choline mono oxygenase (*CMO*) and betaine aldehyde dehydrogenase (*BADH*). This metabolic reaction is triggered by stress. *BADH* and *CMO* are the genes that encode for the enzymes of glycine betaine synthesis and potentially can be used for genetic engineering of wheat to produce transgenic crop tolerant to salt stress and drought as well. Transgenic carrot containing *BADH* gene showed 8-fold enhanced activity of *BADH* enzyme, grew 7-fold more and accumulated 50– 54 fold more betaine and was able to grow in 400 mmol/L NaCl thereby exhibiting highest level of salt tolerance reported so far among genetically modified crops[5]. Wheat plants transformed with *BADH* gene grew normally with healthy roots on the medium containing 0.7% NaCl while the control plants have very poor roots and finally died[6].

Present study was designed to incorporate *BADH* gene that encodes for betaine aldehyde dehydrogenase into callus derived from immature and mature embryos of wheat (*Triticum aestivum*) through gene gun and *Agrobacterium* so as to improve its osmotic stress tolerance.

2 Materials and Methods

2.1 Callus induction from immature embryos

Chinese winter wheat variety Shi 4185 was chosen for transformation experiments. Immature seeds (15 days post anthesis) were surface sterilized with 70% ethanol for one minute followed by sterilization with 0.1% $HgCl_2$ for 7 minutes. Afterwards the seeds were washed with copious amount of sterilized water. Immature embryos were removed aseptically using forceps and placed in callus induction medium (MS + 2,4-D 2 mg/L + sucrose 50 g/L + agar 5 g/L) in flasks keeping the scutella side upward. The explants were cultured for 14 days in dark at 24– 25 ℃. Hardened scutella were separated and good calli were sub-cultured for another 14 days on callus induction medium.

2.2 Callus induction from mature embryos

Good and healthy seeds were sorted out, surface sterilized for one minute in 70% ethanol followed by 14 minutes in 0.1% $HgCl_2$ with one drop of commercial Tween 20. The seeds were then washed three times with ample amount of sterilized distil water. The mature embryos were excised aseptically using forceps and placed in callus induction medium (MS + 2,4-D 2 mg/L + sucrose 50 g/L + agar 5 g/L). The embryos were sub-cultured after 14–21 days if needed.

2.3 Transformation through particle bombardment

Calli from immature embryos were used as the target for particle bombardment. The plasmid vector pABH9 (Fig. 1) containing *bar* and *BADH* genes as selectable marker and the gene of interest, respectively, under the control of maize ubiquitine (ubi) promoter and *nos* (nopaline synthatase) terminator sequence supplied by Professor Chen Shouyi from Chinese Academy of Sciences, Institute of Genetics and Developmental Biology, Beijing was used for transformation of calli. Standard protocols were followed for plasmid extraction, preparation of gold micro-carriers and coating of plasmid DNA onto micro-carriers.

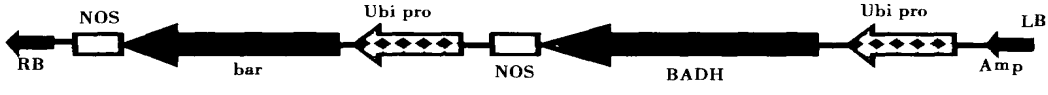


Fig. 1 Diagram of pABH9 plasmid

The calli exhibiting good growth were placed in a circle in the center of the petridishes corresponding to the area that would be blasted with DNA coated gold particles on high osmotic medium containing 0.4 mol/L manitol 8 hours before biolistic bombardment. The petridishes were placed in the vacuum chamber of the gene gun and bombarded with PDS-1000/He (BioRad USA) particle device according to the manufacturer's instructions with a target distance of 6 cm at 1100 psi helium pressure. After three days the calli were transferred to selection medium (MS + 2,4-D 1mg/L + sucrose 30 g/L + agar 5 g/L + 1, 3 or 5 mg/L PPT). NaCl 150 mmol/L alone and 100 mmol/L with 3 or 5 mg/L PPT was used as a selection agent. The calli were sub-cultured after 14 days followed by another subculture but with 1 mg/L 2,4-D for further 14 days. The calli that exhibited strong growth were transferred to regeneration medium (MS + KT 1 mg/L + sucrose 30 g/L + agar 6 g/L). Calli were sub-cultured in regeneration medium after 14–21 days if considered neeessary. Spermidine (1 mmol/L) was used in the regeneration medium to induce shooting from more than 56 days old calli (Khanna and Daggard, 2003) which failed to produce shoots.

2.4 *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens strain AGL1 with plasmid pBin 438 containing *BADH* and NPTII genes, both under the control of CaMV35S promoter and nos terminator (Fig. 2) was used as a biological vector for the gene transfer in these transformation experiments. Calli both from mature and immature embryos were used as the target for the transformation. Bacterial culture was prepared from the 15% glycerol stock. YEB medium was used for culturing of *Agrobacterium* supplemented with 50 mg/L kanamycin, 50 mg/L streptomycin and 25 mg/L rifampicin. The culture was incubated at 27.5 ℃ with shaking (250 r/min) for overnight. When the culture was at log phase or immediately thereafter the cells were pelleted by centrifugation at 5 000 r/min for 2 minutes. The bacterial pellet was suspended in 1/5 MS liquid medium to OD_{600nm} 0.5–1.0 Acetosyringone (100 μmol/L) was also added in the inoculation medium just before the inoculation of callus.

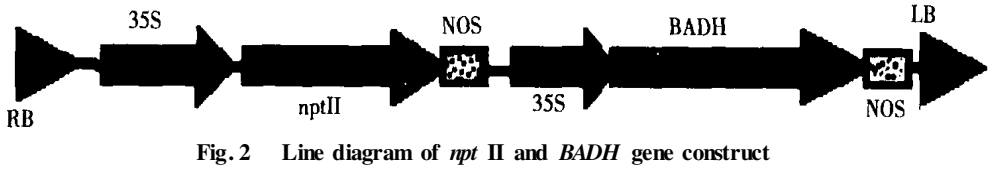


Fig. 2 Line diagram of *npt* II and *BADH* gene construct

The callus was inoculated by submerging in the inoculation medium for 15 minutes, dried on sterilized filter papers and put in the petridishes fitted with filter papers soaked with 1/5 MS salts and 100 μmol/L acetosyringone. Co-cultivation was carried out for three days in the dark at about 25 ℃. After co-cultivation the callus was washed with 500 mg/l cefotaxime sodium antibiotic to kill the bacteria attached to outer surface of explants and placed on selection medium (MS + 2, 4-D 2 mg/L + sucrose 30 g/L + agar 5 g/L) containing 250 mg/L cefotaxime sodium to control the growth of *Agrobacterium*. NaCl 150 mmol/L was used as a selection chemical. The callus was sub-cultured after every 14 days but with 125 mg/L cefotaxime sodium. For the last 14 days of subculture the level of 2, 4-D used was decreased to 1 mg/L. After 42–56 days of selection the callus exhibiting strong growth was transferred to regeneration medium (MS + 2, 4-D 0.5 mg/L + KT 0.2–1.0 mg/L + NaCl 100 mmol/L + sucrose 30 g/L + agar 5 g/L) and put in the growth chamber at 16/8 hours light/dark period at 24–25 ℃. Spermidine (1 mmol/L) was included in the regeneration medium to induce shoots from more than 56 days old calli.

2.5 Screening of transformed plants

The calli that started regenerating were transferred to MS medium solidified with agar without any growth regulator. The regenerated seedlings were washed thoroughly with distilled water, planted in petridishes filled with vermiculite and put in chiller for vernalization. After 23–25 days of vernalization the seedlings were transferred to pots in green house.

Putative transformants obtained through particle bombardment and *Agrobacterium* inoculation were evaluated by the presence of the transgene in the genomic DNA by PCR analysis. DNA for PCR test was extracted by CTAB method. Genomic DNA was amplified using specific primer sequences for *BADH* coding region (as shown below) to amplify a 1.061 kb and 0.901 kb fragments of this gene from the genome of transformed plants through gene gun (1) and *Agrobacterium* (2), respectively. DNA from non-transformed check plant and plasmids (pABH9, pBin438) was used as check to compare amplification of transgene in the genomic DNA. The PCR products were run on 1% agarose gel. The resolved PCR products were visualized on UV trans-illuminator and photographed.

1) 18mer *BADH* F: 5′ > TTC CTG CTC GTC AAC TCT < 3′
18mer *BADH* R: 5′ > CCC TTC ACT CTT CGC TGT < 3′
2) 25mer *BADH* F: 5′ > GAG GAA CAA AGG CAG AGA TTG GGC T < 3′
21mer *BADH* R: 5′ > TGC CCC TTC ACT CTT CGC TGT < 3′

PCR program consisted of an initial denaturation for 10 min. at 94 ℃ followed by 94 ℃ for 1 min, 62 ℃ for 1:30 min, 72 ℃ for 1:30 min. (40 cycles) and 72 ℃ for 10 min. The PCR positive plants obtained through particle bombardment were also analyzed by phosphinothricin (PPT) leaf paint assay for the expression of *bar* gene. A solution of phosphinothricin (3 mg/L) and 0.1% Tween 20 was applied to leaf sections for three times a week at a two-day interval. Absence of excessive necrosis or bleaching as compared to control was considered an indication for integration and expression of bar gene in these plants.

PCR positive plants were exposed to 0.5% (w/w) level of NaCl for the determination of osmotic stress resistance of transformed plants. The plants that were able to grow normally and did not exhibit severe wilting and stunted growth as compared with check plants were assumed to be resistant to this level of osmotic stress. Plants of the same age were used as check.

3 Results

3.1 Gene gun mediated transformation

From the bombardment of 2 933 calli, 473 good calli (16.13%) exhibiting strong growth were selected (Tab. 1) and transferred to regeneration medium for induction of shoots. Use of 1, 3, and 5 mg/L PPT did not show any specific trend in its effect on the selection of transformed calli. Use of 150 mmol/L NaCl alone proved the most effective selection chemical in selection of transformed calli with *BADH*. Only 13% calli were able to proliferate and grow well in the presence of 150 mmol/L NaCl.

Tab 1 Frequency of Gene gun Transformation

Gene	No. of calli bombarded	Selection chemicals	No. of calli selected	Seedlings regenerated	PCR$^+$
1-*BADH*	120	PPT (1 mg/L)	24 (20.0%)	4 (16.60%)[a]	–
	110	PPT (3 mg/L)	21 (19.1%)	4 (19.00%)	–
	120	PPT (5 mg/L)	23 (19.2%)	5 (21.70%)	–
2-*BADH*[b]	90	PPT (1 mg/l)	18 (20.0%)	6 (33.13%)	1
	90	PPT (3 mg/L)	20 (22.0%)	2(10.00%)	–
	80	PPT (5 mg/L)	19 (23.7%)	2 (10.50%)	2
3-*BADH*	386	PPT (3 mg/L)	83 (21.5%)	7 (8.40%)	–
BADH	365	PPT(3 mg/L) + 100 mmol/L NaCl	56 (15.3%)	–	–
BADH	380	PPT(5 mg/L) + 100 mmol/L NaCl	54 (14.2%)	–	–
4-*BADH*	1 192	150 mmol/L NaCl	155 (13.0%)	15 (9.67%)	2
Total	2 933		473(16.13%)	45 (9.51%)	5 (0.17%)[C]

Note: a. Percentage calculated on the basis of selected calli; b. 14 days old calli from immature embryos were used for transformation; c. Net transformation efficiency based on total number of calli bombarded

Initiation of shoots from calli started after 42 – 56 days in regeneration medium. Overall forty-five plant-lets (9.51%) regenerated from 473-selected calli. The net regeneration rate based on total number of bombarded calli from these experiments was 1.53%. The calli produced green spots but instead of producing shoots produced roots in most of the cases. In some cases the calli stopped proliferating, turned brown and died without producing shoots. The regenerated plants were quite normal without any sign of abnormality. Five regenerated plant-lets were found PCR positive (Fig. 3, A-C). Net transformation efficiency based on total number of calli bombarded was 0.17%. PPT-leaf painting confirmed the transformed status of PCR positive plants. Application of PPT paint solution did not produce severe necrotic spots or bleaching of the transformed plants compared with check plant leaf (Fig. 3, D). PCR positive plants were able to thrive in 0.5% NaCl without any stunting or wilting as compared with check plants (Fig. 3, H).

3.2 *Agrobacterium* mediated transformation

Transformation frequency through *Agrobacterium* is presented in Tab. 2. When callus derived from mature embryos was used for inoculation 17.53% good growing calli were selected out of which only 5 seedlings regenerated. Net regeneration rate based on total number of mature calli inoculated was 0.25%. None of the regenerated plant-lets was PCR positive. DNA from randomly selected 20 calli was extracted and subjected to PCR amplification for *BADH*. Four calli (20%) exhibited positive amplification for *BADH* gene (Fig. 3, G). So it explicitly reveals that *Agrobacterium* successfully delivered T-DNA into calli.

Tab 2 Frequency of *Agrobacterium*-mediated transformation

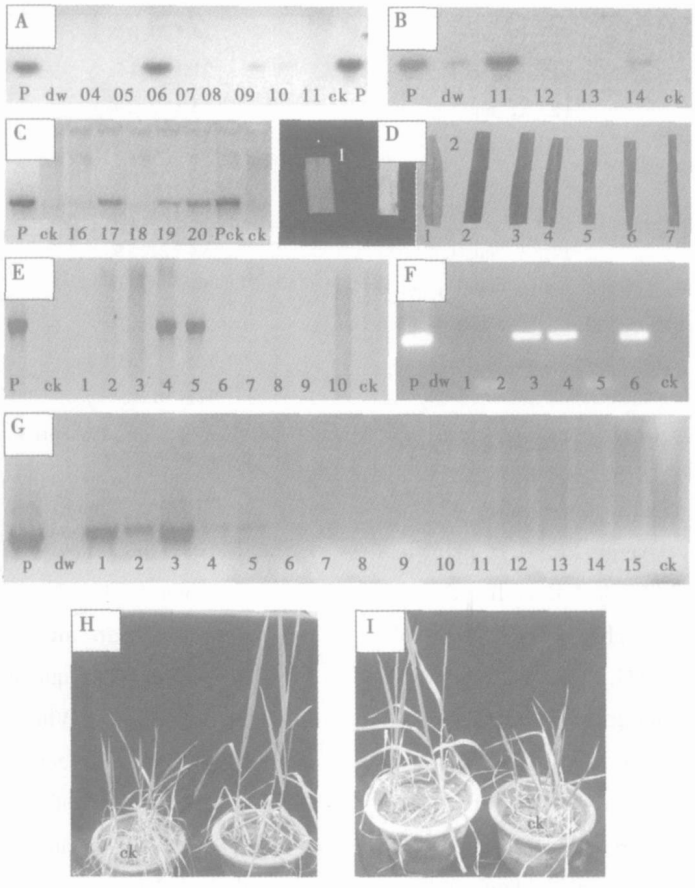
	Selection chemical	Number of calli inoculated	Number of calli selected	Seedlings regenerated	PCR$^+$ plants
1[a]	NaCl 100mmol/L	689	110 (7.66%)[c]	2 (1.82%) –	–
	NaCl 125mmol/L	262	51 (19.46%)	–	–
	NaCl 150mmol/L	1017	184 (21.44%)	3 (2.80%)	–
2[b]	NaCl 150mmol/L	2933	242(14.69%)	21(1.24%)	5
Total		4901	587 (12.09%)	26 (4.43%)	5 (0.10%)[d]

Note: a. Callus induced from mature embryos; b. Callus induced from immature embryos; c. Percentage calculated on the basis of selected calli; d. Net transformation efficiency based on total inoculated call

From inoculation of 2 933 calli derived from immature embryos 21 plant-lets regenerated from 242 selected calli with net regeneration rate of 0.72% based on total number of transformed calli. Integration of *BADH* transgene was confirmed through PCR only in five plant-lets (Fig. 3, E – F). PCR positive plants were able to thrive in 0.5% NaCl without any stunting or severe wilting as compared with check plants (Fig. 3, I).

Use of spermidine in regeneration medium after 56 days could not induce shoot regeneration even after 84

days. Lowering the level of 2, 4-D from 2 to 1 mg/L 14 days before transferring to regeneration medium and variation in the level of KT also did not prove fruitful for regeneration.



A- C. PCR amplification for 1.061 kb fragment of *BADH* gene from genomic DNA of plants transformed through gene gun. Sample number 6, 11, 17, 19 and 20 show positive amplification corresponding to plasmid (pABH9) amplification; D. Effect of PPT leaf painting on transformed and check plants. Left: difference in transformed and check plant, Right: variable response of leaves from transformed plants compared with check plants. 1-check plant with PPT leaf painting, 2-check plant without PPT leaf painting, 3- 7-transformed plants with PPT leaf painting; E, F. PCR amplification for 0.901 kb fragment of *BADH* gene from genomic DNA of plants transformed through *Agrobacterium*; G. PCR amplification for *BADH* from DNA extracted from randomly selected calli from selection medium inoculated with *Agrobacterium*; H, I. Effect of NaCl (0.5%) on check plants and plants transformed through gene gun (H) and *Agrobacterium* (I)

Fig. 3 PCR test, PPT leaf painting and salt tolerance of transformed plants through gene gun and *Agrobacterium*.

4 Discussion

4.1 Gene gun and *Agrobacterium* mediated transfer of BADH gene

Selection of transformed callus is very crucial for transformation. This is the basic screening of transformed cells from non-transformed ones. It mainly depends upon the efficacy of selection chemical used for this purpose. In these experiments when *BADH* gene was transferred with bar selectable marker phosphinothricin (PPT) was used as a selection chemical. Different concentrations of the chemical were not found to differ significantly in selection of calli. This selection system does not seem to work effectively in checking the growth of calli that do not integrate and express the transgene. Vasil[7] also reported the growth of non-transformed calli when PPT was used as a selection agent. Use of NaCl alone or with PPT proved relatively more effective selection chemical. Age of calli had a significant effect on the regeneration capability from the transformed calli. Short duration of culture can maintain the regeneration potential of callus for embryogenesis[8], so 14 days of culture before bombardment resulted in relatively higher regeneration rate.

Selection escape rate was very high in these experiments. This possibly may be due to ineffectiveness of the selection system. Different levels of PPT and NaCl could

not check the growth of non-transformed calli thereby leading to high selection escape rate. Other investigators have reported selection escapes. Nehra *et al*[9] faced this problem and found that 50% plants were not transformed when selected on 5 mg/L PPT. Selection escapes with PPT selection were also reported by Rasco-Gaunt *et al*[10], but no selection escapes were found by Patnaik and Khurana[11] with the use of PPT during the transformation of wheat.

The net transformation efficiency is a good indicator for the evaluation of the transformation system. The net transformation efficiency based on the total number of bombarded calli was 0.17%. Wheat has been transformed successfully using particle bombardment[11,12] with transformation efficiency ranging from 0.1% to about 9%. Low efficiency of transformation of these experiments is mainly because of low regeneration of green plantlets from the transformed calli. Low transformation efficiency also manifested that the particle bombardment system is not very efficient in producing transgenic plants on large scale. Its maximum efficiency is not more than 5%[13]. Several factors may be ascribed responsible for low transformation efficiency but regeneration response of the genotype is the major factor. Most of the calli in these experiments did not produce shoots even after 70 days. This was in contradiction to the findings of some investigators who reported the regeneration of calli after 21 days in regeneration medium[7,11].

Leaf painting with PPT-paint solution did not cause bleaching of the transformed leaves. This conforms to PCR amplification of the transformed plants for *BADH* gene. The non-transformed check plant leaves exhibited bleaching or severe necrosis. This indicates the reliability of *bar* selectable marker for transformation of wheat that has been used with success by several investigators[11,14]. But use of PPT in the selection medium could not prove very effective so the selection strategy with PPT applied in these experiments does not seem to work effectively. The PPT-based selection system needs to be modified. Increasing the level of PPT in selection medium and inclusion of PPT in the regeneration medium to avoid the regeneration of non-transformed plantlets may prove better.

Wheat has been successfully transformed through *Agrobacterium*[12,15,16], but reports to transform wheat through *Agrobacterium* are less than that from particle bombardment. This is mainly due to the influences of *Agrobacterium* to the regeneration system. With the development of reliable protocols for genetic transformation of wheat *Agrobacterium* is becoming a method of choice. The choice of explant for successful *Agrobacterium* mediated transformation is very crucial for wheat. Presently callus derived from immature embryos is the most preferred[15,17] explant for callus-based transformation through *Agrobacterium*. Callus derived from both mature and immature embryos was used as an explant source for these experiments. It was found that *Agrobacterium* successfully delivered T-DNA containing *BADH* gene into the callus derived from immature and mature embryos that was indicated from the PCR test of the randomly selected calli from the selection medium. So callus from mature embryos is also a suitable host and equally competent for incorporation of the transgene through *Agrobacterium*. Although use of immature embryos is better than mature embryos for gene gun and *Agrobacterium* mediated transformation but protocol improvements and modifications in medium composition for enhancing shoot regeneration from mature-embryos-derived callus will help to continue the *Agrobacterium*-mediated transformation throughout the year with out any special requirement for the explant source. Mature embryos are available throughout the year for induction of callus and transformation process can be continued without green house that is required for the supply of immature embryos year round. Callus derived from mature embryos of wheat was successfully used for transformation through particle bombardment[11]. Low rate of plant regeneration is the main hurdle for genetic improvement of wheat through genetic engineering. Transformation efficiency through *Agrobacterium* may be much higher if cultural conditions are improved to avoid growth of *Agrobacterium* and for better regeneration of green plantlets. *Agrobacterium* is a good vector for the transfer of alien genes into wheat but low regeneration rate limits the production of significant number of transformed plants. Selection agent used in these experiments was NaCl. This seems to be an effective chemical in the selection of transformed calli. The selection with NaCl is important as this can help to

avoid the use of selectable markers for transfer of stress tolerance genes such as *BADH*.

Low regeneration of wheat seedlings from transformed callus has been the usual limitation[18]. Long period of *in vitro* culture may be ascribed responsible for the loss of embryogenic potential of the callus. Transformation of callus after short period of callus induction may be a good strategy to avoid the loss of regeneration capability of the callus. In these experiments when the total culture duration was short the regeneration was better. The color of the callus changes to brown indicating the loss of ability for regeneration. Long period of callus phase leads to significant reduction in morphogenetic potential. Spermidine has been found to promote the morphogenetic potential of old callus from immature wheat embryos. Therefore, regeneration medium was supplemented with spermidine to induce regeneration of seedlings but met with no success although it has been reported to improve the regeneration from the transformed calli of wheat[19] and sorghum(Sargent *et al*). This may be due to poor response of wheat to *in vitro* culture with little regeneration. Most of the calli in these experiments did not produce shoots even after 70 days. Lowering the concentration of 2, 4-D from 2 to 1 mg/L in the selection medium before transferring to regeneration medium also did not improve regeneration contrary to the findings of Rasco-Gaunt *et al*[10] who were able to enhance somatic embryogenesis and regeneration with lowering the level of 2, 4-D 14 days before transferring to regeneration medium. Variation in the level of KT also could not affect induction of shoots. This obviously reveals that callus based transformation is dependent on the response of genotype to *in vitro* culture and callus intermediate of this system is main hurdle for mass production of transgenic wheat plants. Wheat is usually considered the most recalcitrant species to *in vitro* culture with little regeneration.

4.2 *BADH* transgene and osmotic stress tolerance

BADH gene was successfully incorporated and expressed in wheat plants through particle bombardment and *Agrobacterium*. The transformed plants were able to tolerate osmotic stress due to presence of 0.5% (w/w) NaCl in the growth medium. *BADH* gene has been successfully incorporated and expressed in wheat for regulation of metabolic pathways and osmotic stress resistance of plants[2,6,20] through particle bombardment. Insertion of *BADH* gene into crop plants has shown a significant improvement in the response of plants to salt stress. *BADH* gene was transferred through *Agrobacterium* and expressed in tobacco[21] and tomato[22]. The transformed plants were found to tolerate various levels of salinity. Generation of salt tolerance by introduction of alien genes into plants to alter their metabolic response for osmoregulation in salt stress is a better strategy than conventional breeding approach. Enhanced production of glycine betaine aldehyde dehydrogenase enzyme due to expression of *BADH* transgene can cause significant increase in the production of glycine betaine that is then responsible for the protection of enzyme activity and better performance of transformed plants under osmotic stress[5]. *BADH* is also found to play a role in drought resistance of plants. Drought tolerance of Bermuda grass[23] and sorghum[24] when transformed with *BADH* was much better than control plants. Therefore, production of transgenic wheat by incorporation of *BADH* gene for salt resistance will also enable the crop to tolerate drought conditions.

5 Conclusion

Gene gun and *Agrobacterium* were successfully employed for the insertion of *BADH* gene into wheat callus. Low transformation efficiency was mainly due to poor regeneration of transformed calli into seedlings. *Agrobacterium* is more suitable delivery system in terms of ease with which it can be used for transformation and operational cost as compared with gene gun but its influence on cultures should be considered. Mature embryos are equally competent for transformation. Improvements in callus induction and regeneration procedures from mature embryos derived callus will make the transformation of wheat convenient throughout the year with out any special requirement.

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