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Research Article

Screening of *ZAT12* and *GlyII* wheat transgenics using seedling based assays for stress tolerance

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Abstract

Single copy transgenics of *Agrobacterium* mediated wheat transformants were available for two genes- *Zat12* and *GlyII* in the background of wheat cultivar DPW 621-50. Three homozygous transgenic lines carrying *ZAT12* and three carrying *GlyII* were subjected to preliminary screening for water deficit and salinity stress respectively. An initial experiment was carried out to standardize the concentrations of PEG and NaCl using parental non- transformed line DPW 621-50. Seedlings were grown in vermiculite in propagation trays in a growth chamber maintained at $20^0C \pm 2^0C$, 8/16 hours dark/light *ZAT 12* homozygous progenies were screening using PEG at 25% and 30% concentration. Transgenics *Z-8-12* and *Z-15-10* showed significant improvement for shoot and root traits under PEG induced water deficit stress. In the best transgenic line, 45%, 20%, 33%, 22%, 24%and 20% improvement over DPW 621-50 was observed for root length, shoot length, fresh root weight, root dry weight, shoot fresh weight and shoot dry weight respectively under stress conditions. In a parallel experiment, all the three *GlyII* transgenics showed marked superiority over parental cultivar DPW 621-50. The best *GlyII* transgenic showed 25%, 19%, 12%, 22%, 26% and 22% improvement over PBW 621 for root length, shoot length, fresh root weight, root dry weight, shoot fresh weight and shoot dry weight respectively under stress conditions.

Keywords: *Agrobacterium,* water deficit stress, salinity stress, transgenics, DPW621-50

1. Introduction

Declining ground water and spread of salinity are becoming particularly important for wheat and are likely to impinge on national food security. Response options include development of stress resistant crop varieties, particularly in case of major food crops. Conventional breeding strategies, however, have proved relatively more effective for resistance to biotic stresses as compared to abiotic stresses. The progress of conventional breeding for abiotic stress tolerance is slowed down in part due to the limited availability of suitable genetic variation for breeding. Genetically modified crops hold potential for enhancing abiotic stress tolerance in crops and with increasing availability of cloned and well characterized genes may emerge as a major practical strategy, once biosafety concerns are properly addressed. Many of the genes known to be involved in stress tolerance have been isolated initially in model plant species such as Arabidopsis. Study of experimental transgenics involving a variety of potentially useful genes is an important prelude to commercial deployment of stress tolerant transgenic crops. Two such transgenics carrying ZAT12 or GlyII gene in background of wheat variety DPW 621-50 were taken up for the present study.

Zinc-finger protein gene *ZAT12* is a representative of the small group of genes that are involved in transcription regulation during stress. *Zat12* was found to respond at the steady-state transcript level to ozone fumigation, wounding, heat, cold and drought. Further, *Zat12* is expressed in roots, flowers, and developing seeds, tissues associated with expression of stress-response genes. *Zat12* gene isolated from *Arabidopsis thaliana* when constitutively expressed in *Arabidopsis* transformants (Davletova *et al* 2005, Vogel *et al* 2005) and *Zat12* from *Brassica carinata* when transferred to tomato (Chandra *et al* 2013) conferred tolerance to different abiotic stresses. Analysis of relative water content, electrolyte leakage, chlorophyll colour index, H_2O_2 level and catalase activity suggested that tomato *Zat12* transformants had significantly increased levels of drought tolerance (Chandra *et al* 2013). Shah *et al* (2013) demonstrated that *BcZat12* transformed tomato over-expressing the gene product was tolerant to heat-shock (HS)-induced oxidative stress.

Glyoxalase enzymes are important for the glutathione (GSH) based detoxification of methylglyoxal, which is formed primarily as a byproduct of carbohydrate and lipid metabolism. Methylglyoxal (MG) is a potent mutagenic and cytotoxic compound known to arrest growth, react with DNA, protein and increase sister chromatid exchange . MG concentration varies in the range of 30-75 micromole in various plant species and it increases 2 to 6-fold in response to salinity, drought, cold stress condition (Yadav *et al* 2005). *Glyoxalase I* catalyses the formation of lactoylglutathione from the hemithioacetal formed non-enzymically from methyl glyoxal and reduced glutathione. *Glyoxalase II* (*GlyII*) catalyses the hydrolysis of lactoylglutathione to lactic acid and regenerates the reduced glutathione consumed in the *glyoxalase I* catalysed reaction . Several studies have indicated that *GlyII* enzyme may have an important and independent role in conferring salt stress tolerance. The over expression of glyoxalases could enhance the level of reduced glutathione that presumably helps to detoxify reactive oxygen species which in turn can result in tolerance against salt stresses (Singla-Pareek *et al* 2003). *GlyII* gene has been isolated from *O. sativa* and transferred to tobacco (Singla-Pareek *et al* 2003, Singla-Pareek *et al* 2006), rice (Singla-Pareek *et al* 2008, Wani and Gosal 2011) and *Brassica* (Saxena *et al* 2011). Several of the transformants in these studies exhibited enhanced salinity tolerance.

Agrobacterium mediated wheat transformants (T_0) were developed for two genes- *Zat12* and *GlyII* in the background of wheat cultivar DPW 621-50 (Kaur 2014) at PAU, Ludhiana. The transformants were advanced to T_3 while transgene inheritance was monitored using gene based (*ZAT12* and *GlyII*) and gene construct based (CaMV35S promoter and antibiotic resistance gene *nptII*) PCR primers. A set of homozygous transgenic lines carrying either *ZAT12* or *GlyII* were subjected in the present study for preliminary screening to access water deficit and salinity stress respectively.

2. Materials and methods

An initial experiment was carried out to standardize the concentrations of PEG and NaCl using parental non-transformed line DPW 621-50 (Also known as PBW 621). The methodology followed was the same as explained in the following paragraphs for shortlisted concentrations of PEG and NaCl used in actual screening.

In the first experiment with transformed material, three *ZAT12* homozygous T3 lines Z-8-12, Z-8-19 and Z-15-10 along with wheat cultivar DPW 621-50 were evaluated. Seedlings were grown in vermiculite in propagation trays. The seeds of the progenies were sown in three sets of propagation trays- one serving as control and the other to which stress was administered in two doses- 25% PEG and 30% PEG. The experiment was conducted in a growth chamber maintained at $20^0C \pm 2^0C$, 8/16 hours dark/light. These three sets of propagation trays were irrigated with ¼ MS media for the first seven days (normal irrigation). From eighth day onwards (when seedlings were 5-7 cm long), 25% and 30% poly ethylene glycol (PEG) solution in ¼ MS media were respectively used as the moisture stress inducing media. Both sets of propagation trays were individually immersed into another shallow tray containing 2.5 litres of 25% and 30% PEG solution (in ¼ MS media), and kept as such for 2 hours (to allow sufficient time for roots to imbibe solution), whereas, the third set maintained as control, was immersed into another shallow tray containing 2.5 litre of ¼ MS media. Thereafter, every alternate day, two sets were respectively irrigated with ¼ MS media (control) and 25% and 30% PEG solution in ¼ MS media (stressed) till next ten days. Thus, after 17 days of seeding, data was recorded for the below listed growth parameters.

In a parallel experiment, seeds of three *GlyII* positive progeny G-1-13, G-2-2 and G-3-4 along with wheat cultivar DPW 621-50 were sown in three sets of propagation trays one serving as control and the other to which stress was administered in two doses- 300mM NaCl and 400mM NaCl. The experiment was conducted in a growth chamber maintained at 20^0C ± 2^0C, 8/16 hours dark/light. Seedlings were grown in vermiculite in propagation trays. These three sets of propagation trays were irrigated with ¼ MS media for the first seven days (normal irrigation). From eighth day onwards (when seedlings were 5-7 cm long), 300mM and 400mM NaCl solution in ¼ MS media were respectively used as the salt stress inducing media (Blum *et al* 1980). Both sets of propagation trays were respectively immersed into another shallow tray containing 2.5 litres of 300mM and 400mM solution (in ¼ MS media), and kept as such for 2 hours (to allow sufficient time for roots to imbibe solution), whereas, the third set maintained as control, was immersed into another shallow tray containing 2.5 litre of ¼ MS media. Thereafter, every alternate day, two sets were respectively irrigated with ¼ MS media (control) and 300mM and 400mM NaCl solution in ¼ MS media (stressed) till next ten days. Thus observations were recorded after 17 days of seeding.

Observations in case of both experiments were recorded on following traits:

2.1 Root length: Root length was measured with a scale (ten seedlings per treatment from three replications) from tip of the crown to the maximum length and expressed in cm.

2.2 Shoot length: Shoot length was measured with a scale (ten seedlings per treatment from three replications) from tip of the crown to the maximum length and expressed in cm.

2.3 Root and shoot fresh weight: The root and shoot of ten seedlings (used for measuring length) were respectively pooled within each of the three replicates and fresh weight of the pooled root and pooled shoot samples (in triplicate) was determined and expressed as mg/ ten seedlings fresh weight.

2.4 Root and shoot dry weight: Fresh tissue of the root and shoot samples was subjected to drying at 60^0C for 72 hours in oven and then the dry weight of the respectively pooled root and shoot samples was determined and expressed as mg/ ten seedlings dry weight.

Observations were subjected to analysis of variance to see if transformed material out performs the parental line for the above traits under stress conditions.

3. Results and discussion

3.1 Standardization of concentrations of stress causing compounds

Polyethylene glycol in high concentrations creates water stress conditions, providing a convenient screening method for drought stress tolerance. To devise appropriate screening methodolgy PEG concentration of 20%, 25%, 30%, 35% were used with non transformed seedlings of DPW 621-50. Clear cut difference in root and shoot morphology were observed at different PEG concentration (Table 1, Figure 1).Maximum reduction in root length and shoot length occurred at 35% PEG concentration At this concentration root length was 4.02 cm whereas in control it was 11.44 cm. In 20% PEG less drastic reduction were observed (length 8.72cm). In 25% PEG root length was 7.24 while in 30% PEG concentration it was 4.84cm. In case of shoot, length under non stressed condition was 14.28cm. Shoot length in 25% PEG was 12.24cm and in 35% PEG length was further reduced to 8.96cm. Similar trend in response to PEG concentrations was observed for root and shoot fresh weight and dry weight. Based on these observation 25% and 30% PEG was selected for screening of transgenic material using propagation trays.

Table 1. Response of PBW 621 seedlings to different concentrations of PEG

Parameter	Control	20% PEG	25% PEG	30% PEG	35% PEG
Root Length(cm)	11.44	8.72	7.24	4.84	4.02
Shoot Length(cm)	14.28	12.75	12.24	10.38	8.96
Root fresh weight(mg)	150	90	65	45	35
Root dry weight(mg)	35	25	14	10	5
Shoot fresh weight(mg)	200	130	95	58	32
Shoot dry weight(mg)	30	23	18	11	8

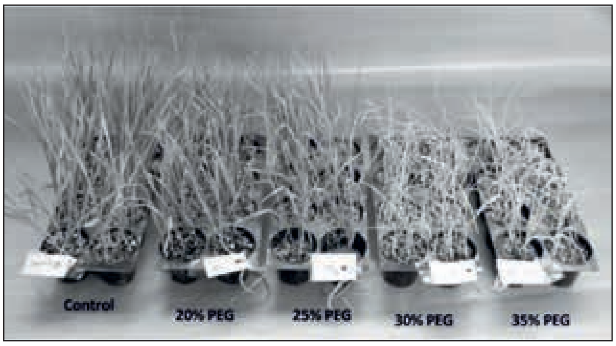

Fig. 1 Response of PBW 621 seedlings to different PEG concentrations

For standardizing the concentrations to be used for screening of GlyII transformed material, nine NaCl levels i.e., 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM were evaluated using parent line DPW 621-50 (Table 2,Figure 2). On the basis of root length, shoot length, root and shoot fresh weight and dry weight 3 concentrations i.e., 200mM, 300mM, 400mM were chosen for screening purpose .

Table 2. Response of PBW 621 seedlings grown in propagation trays to differentconcentrations of NaCl

Concentration of NaCl	Root length (cm)	Shoot length (cm)	Root fresh weight (mg)	Root dry weight (mg)	Shoot fresh weight (mg)	Shoot dry weight (mg)
Control	20.12	22.4	105.4	55	180	91
50 mM	19.3	21.5	95.5	42.1	165	85
100 mM	18.1	20.92	86.1	33.5	148	75
150 mM	17.6	18.9	59.8	29.8	135	62
200 mM	12.5	15.6	33.5	22.9	95	43
250 mM	8.46	12.5	27.6	17.5	61.8	31.5
300 mM	7.1	10.7	18.4	12.4	52.8	24.9
350 mM	6.1	10	16.7	8.1	31.6	15.8
400 mM	5.6	8.32	15.1	6.5	23.8	12.1

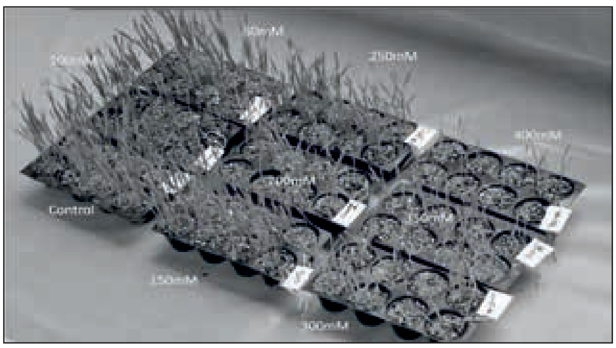

Fig. 2 Seedling growth of PBW 621 under different concentrations of NaCl

3.2 Screening of ZAT12 transgenics for PEG induced water deficit stress

In case of ZAT12 gene, three homozygous lines were screened at two PEG concentrations (Table 3, Figure 3). Root length observations in 25% PEG and 30% PEG solutions showed small but significant difference between non transgenic DPW621-50 and *ZAT12* positive plants. In 25% PEG both Z-15-10 and Z-8-19 had significantly increased root length compared to PBW621, but at higher concentration (30% PEG) only Z-15-10 showed significantly increased root length. One genotype, Z-8-12, at both concentrations, was statistically at par with PBW621.

Table 3. Seedling parameters of *Zat12* transformed lines and DPW621-50 under PEG induced water stress.

Genotype	Root Length(cm)			Shoot Length(cm)		
	25% PEG	30% PEG	CONTROL	25% PEG	30% PEG	CONTROL
Z-8-12	8.16	6.0	13.83	11.36	7.52	20.27
Z-8-19	8.50	6.3	14.20	12.33	7.93	20.80
Z-15-10	9.13	6.9	14.00	12.63	8.30	21.10
PBW621	8.067	6.0	14.03	11.37	7.63	20.57
CD(0.05)	0.253	0.345	NS	0.58	0.29	0.35
	Root fresh weight(mg)			Shoot fresh weight(mg)		
	25% PEG	30% PEG	Control	25% PEG	30% PEG	Control
Z-8-12	71.5	38.53	153.37	12.40	8.36	20.20
Z-8-19	73.63	40.1	152.47	12.97	8.37	22.10
Z-15-10	74.47	41.13	152.9	13.50	8.82	22.67
PBW621	72.3	38.83	152.27	12.47	8.13	21.33
CD(0.05)	1.037	1.335	NS	0.48	0.35	1.21
	Root dry weight(mg)			Shoot dry weight(mg)		
	25% PEG	30% PEG	Control	25% PEG	30% PEG	Control
Z-8-12	106.0	56.50	196.17	15.70	11.40	25.13
Z-8-19	107.1	60.10	200.17	16.20	12.93	25.53
Z-15-10	108.4	60.73	200.53	16.60	13.30	25.70
PBW621	105.1	57.60	198.60	15.63	11.70	25.73
CD(0.05)	0.871	1.21	2.51	0.374	0.462	NS

Fig. 3 Response of different T_2 plants for 25% PEG concentrations

In stressed condition two genotypes i.e. Z-8-19 and Z-15-10 were significantly different from DPW621-50. They showed increase in shoot length under stressed condition. In both 25% and 30% PEG concentration these two genotypes have better performance than PBW621. On the contrary Z-8-12 had mean shoot length similar to PBW621. In 30% PEG it showed less growth than PBW621. Overall this genotype is statistically at par with DPW621-50.

It was found that in non stressed condition there was no difference in root fresh weight of transgenic group and PBW621. In stressed condition two ZAT12 positive transgenics, Z-8-19 and Z-15-10, showed less reduction in fresh weight as compared to PBW621

In stressed condition Z-8-19 and Z-15-10 had dry root weight of 12.96mg and13.5mg respectively which was slightly higher than PBW621(12.45mg). In 30% PEG severe reduction in root growth was observed. In this case only Z-15-10 had higher dry weight than PBW621 whereas other two transgenic genotypes were statistically at par with PBW621.

The three transgenics showed better performance than DPW621-50 for shoot fresh weight. On increasing stress to the next level it was found that out of three two transgenic genotypes i.e. Z-8-19 and Z-15-10 performed better than DPW621-50.

For dry shoot weight it was found that under non stressed condition there was no significant difference between PBW621 and transgenics. Under25% PEG stress root dry weight was reduced drastically. Two transgenics i.e., Z-8-19 and Z-15-10 had better performance than DPW621-50. Under 30% PEG aforementioned transgenics had significantly more dry weight than DPW621-50.

Screening of GlyII transgenics under salinity stress

Three homozygous transgenic genotypes denoted as G-2-2, G-1-13, G-3-4 were taken for screening of seedlings raised in micropropagation trays subjected to three NaCl concentrations (Table 4, Figure 4). Transgenics under non stressed conditions showed almost same root length as DPW621-50. Under salt stress root length reduction occurred for both, but was more prominent in DPW621-50. All the transgenics showed significantly better root length than parental line at all stress levels (200, 300 and 400mM of NaCl).

Table 4. Seedling parameters of *GlyII* transformed lines and DPW621-50 under salinity stress

	Root length(cm)				Shoot length(cm)			
Genotype	200mM	300mM	400mM	Control	200mM	300mM	400mM	Control
G-2-2	11.15	10.00	8.70	20.17	14.99	13.27	10.86	20.33
G-1-13	11.52	10.64	7.86	20.59	14.96	13.97	10.37	20.98
G-3-4	10.96	10.41	7.42	20.24	15.48	13.93	10.26	20.89
PBW621	8.46	7.75	6.38	19.90	12.44	10.27	8.05	19.94
CD(0.05)	0.694	0.996	0.641	NS	1.12	0.606	0.978	NS
	Root fresh weight(mg)				Root dry weight(mg)			
Genotype	200mM	300mM	400mM	Control	200mM	300mM	400mM	Control
G-2-2	29.73	21.73	19.87	119.53	15.50	11.56	8.20	21.30
G-1-13	27.47	21.11	19.45	127.33	14.97	10.99	8.03	21.10
G-3-4	31.13	22.13	18.87	118.8	15.03	11.2	7.93	21.30
PBW621	24.20	17.77	17.2	115.33	12.99	8.14	5.43	21.19
CD(0.05)	3.194	1.204	0.638	NS	0.389	0.57	0.44	NS

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	Shoot fresh weight(mg)				Shoot dry weight(mg)			
Genotype	200mM	300mM	400mM	Control	200mM	300mM	400mM	Control
G-2-2	106.80	79.00	38.20	217.63	25.27	19.43	12.10	35.50
G-1-13	104.93	78.47	33.27	225.47	24.83	18.73	11.80	35.37
G-3-4	114.47	83.60	38.27	218.93	25.90	18.93	12.00	35.70
PBW621	88.60	61.73	30.27	211.93	22.73	16.60	9.30	35.80
CD(0.05)	7.17	5.38	1.78	4.73	0.43	0.51	0.42	NS

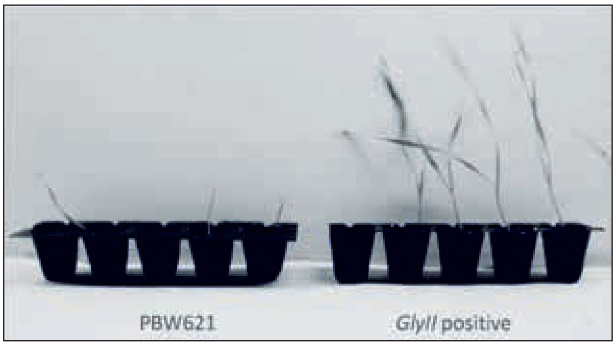

Fig. 4 Effect of salinity stress on *GlyII* positive lines and PBW621 in propagation trays

For shoot length also the above mentioned trend was evident. It was found that under non stressed condition there was no difference between DPW621-50 and the transgenic lines. But when stress was applied it was found that PBW621 was less tolerant to stress in comparison to transgenics. It showed more decrease in root growth than transgenic plant. At 200mM, 300mM, 400 mM PBW 621 showed 57%,61%,68% reduction respectively whereas best performing transgenic G-2-2 at 200mM, 300mM,400mM showed 43%, 50%, 56% reduction respectively.

Unequivocal superiority of the transgenics was observed for both root fresh and dry weight at all salt concentrations whereas no differences were seen under control conditions. An excellent corroboration and consistency of results were evident for both traits.

The shoot fresh weight data of transgenics reveals their clear superiority under stress but inexplicably some genotypic differences for this trait were also obtained under control conditions. Shoot dry weight under stress showed a neat trend of higher tolerance in transgenics while no differences were observed under controlled conditions.

The transgenics used in the present study had a good indication of single gene insertion from the segregation data in T_1 to T_3 generation using multiple gene based molecular markers. Single copy insertions, a hallmark of *Agrobacterium* mediated transformation,are largely free of gene silencing related problems. One of the ZAT12 transgenics Z-8-12 seemed to be less efficient under stress when compared to the other two transgenics. Such differences are often observed across different transformation events While the causes of such variation can be investigated, the best strategy is to have multiple events, which allow for selection of transformant with appropriate trait/gene expression. Another important observation in this study relates to a more clear superiority over parental line DPW 621-50 in case of *GlyII* transgenics when compared with *ZAT12* transgenics. One likely reason can be that *GlyII* has a more specific salt tolerance role while action of ZAT12 is more diffused and covers a wide range of stress situations. It is noteworthy that the transgene does not seem to confer a handicap as indicated by similar responses of transgenics and non transformed parent in most of the non-stress assays.

The initial indication of effective transformation in this material at level of seedling traits is now being followed up by whole plant studies while efforts have also been initiated for pyramiding of the two transgenes in DPW 621-50.

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