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Assessment of nematode resistance in wheat transgenic plants expressing potato proteinase inhibitor (*PIN2*) gene

Dalia Vishnudasan[1], M.N. Tripathi[2], Uma Rao[2] & Paramjit Khurana[1,*]
[1]*Department of Plant Molecular Biology, University of Delhi South Campus, New Delhi 110 021, India*
[2]*Division of Nematology, Indian Agricultural Research Institute, New Delhi 110012, India*

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Abstract

Serine proteinase inhibitors (IP's) are proteins found naturally in a wide range of plants with a significant role in the natural defense system of plants against herbivores. The question addressed in the present study involves assessing the ability of the serine proteinase inhibitor in combating nematode infestation. The present study involves engineering a plant serine proteinase inhibitor (*pin2*) gene into *T. durum* PDW215 by *Agrobacterium*-mediated transformation to combat cereal cyst nematode (*Heterodera avenae*) infestation. Putative T_0 transformants were screened and positive segregating lines analysed further for the study of the stable integration, expression and segregation of the genes. PCR, Southern analysis along with *bar* gene expression studies corroborate the stable integration pattern of the respective genes. The transformation efficiency is 3%, while the frequency of escapes was 35.71%. χ^2 analysis reveals the stable integration and segregation of the genes in both the T_1 and T_2 progeny lines. The PIN2 systemic expression confers satisfactory nematode resistance. The correlation analysis suggests that at $p < 0.05$ level of significance the relative proteinase inhibitor (PI) values show a direct positive correlation vis-à-vis plant height, plant seed weight and also the seed number.

Introduction

Approximately 2500 species of plant-parasitic nematodes cause severe damage and economic losses worldwide amounting to ~$100 billion each year (Sasser & Freckman, 1987). The major plant parasitic nematodes infesting Indian wheat include cereal cyst nematode (CCN- *Heterodera avenae*) and the ear-cockle nematode (*Anguina tritici*). In India, *H. avenae* causes severe damage to cereal crops such as barley and wheat in Rajasthan resulting in heavy economic losses. CCN infestation in wheat root results in decline of root and shoot growth and also a concomitant reduction in transpiration.

Heterodera commonly known as cyst nematode comprises of ~60 species. *H. avenae* is an endoparasitic nematode belonging to the order Tylenchida. Life cycle of *H. avenae* can be divided into four juvenile stages (J1 through J4) and it has only one generation per year, with the hatching of eggs determined largely by temperature. Emergence of juveniles from the cyst depends on the root exudates of the host, sensing which the juveniles pierce the root and move intra- and inter-cellularly

*Author for correspondence
E-mail: paramjitkhurana@hotmail.com

towards the developing vascular tissues by piercing rhizodermal cells with the help of their distinct stylet (Wyss & Zunke, 1986). Juveniles may feed from individual cells as they penetrate through cell walls marked by conspicuous extensive cell disruptions, while migrating to form a permanent feeding site the "syncytium". The cyst nematodes feed from this syncytium and the females complete their life cycle within the root after depositing eggs in the discernible lemon shaped cysts that are resistant to chemical and physical interventions. It is thus imperative to generate a durable and efficient resistance that controls a broad range of plant parasitic nematodes. Methodologies aimed towards the development of transgenic plants with improved nematode resistance encompass strategies such as anti-invasion and migration, feeding-cell attenuation and anti-nematode feeding (Atkinson et al., 1995; 2003; Vishnudasan & Khurana, 2005).

The plant proteinase inhibitors (PI's) bind with the proteinases present in the gut and thereby hinder its proteolytic activity that further impedes the development of feeding pests. Plant PI's are generally categorized according to the class of proteases that they inhibit (Ryan, 1990). There are four types of proteases based on the active amino acid in the active center e.g. – cysteine, serine, metallo- and aspartyl proteases. Some plant serine protease inhibitors (SPI's) are bifunctional, typically possessing trypsin and α-amylase inhibitor activities, while other PI's exist as multidomain proteins. Serine PI's are the most extensively characterized class of plant PI's that function in defense against herbivores (Lawrence & Koundal, 2002). Genes encoding members of various SPI's have been cloned and introduced into transgenic plants and analysed for their effectiveness in controlling insect pests (Johnson et al., 1989; Duan et al., 1996; McManus et al., 1999) as well as the plant-parasitic nematodes (Atkinson, 1993; Hussey, 1993). *Heterodera glycines* expresses three serine proteases, HGSP-I, -II and -III. cDNAs encoding cysteine and serine digestive proteinases localized the major proteolytic activity to the intestine thereby corroborating the possibility of transformants expressing the PIs such as CpTI and oryzacystatin (Oc-I) might be effective against these proteinases (Lilley et al., 1996, 1997; Urwin et al., 1997). PI-based transgenic defences have been accomplished in plants against the plant-parasitic nematodes (Hussey & Grundler, 1998), for example the growth rate of juveniles of potato cyst nematode was reduced and the sex ratio shifted towards more males when CpTI was expressed under CaMV 35S promoter (Hepher & Atkinson, 1992; Hussey, 1993).

The present work evaluates the prospects of a serine PI – *PIN2* towards conferring nematode resistance in transgenic wheat. Therefore in the present investigation a potato proteinase inhibitor (*PIN2* – a serine proteinase inhibitor) gene was introduced into wheat (*T. durum* PDW215) *via* *Agrobacterium*-mediated transformation. The *PIN2* gene ostensibly conferred insect resistance in *japonica* rice (Duan et al., 1996) wherein the expression of the *PIN2* gene was regulated by its own promoter (*Act*1 gene intron present within for enhanced expression levels of the promoter) and 3′ terminator sequence. The expression of the *PIN2-Actin* 1 intron-*gusA* fusion gene displays a systemic wound response in rice (Xu et al., 1993) implicating that certain steps of the wound response signal and the transduction pathways are possibly conserved between dicots and monocots.

Materials and methods

Production of PIN2 *wheat transformants*

Mature embryos of *Triticum durum* var. PDW215 were aseptically isolated along with the scutellar region and placed on MSE2 medium (MS medium + 200 mg/l casein hydrolysate + 100 mg/l myo-inositol) with the embryo axis facing downwards. *Agrobacterium*-mediated transformation with pCAMBIA3301:*PIN2* (Figure 1(a)) was undertaken according to Patnaik et al. (2005). The axenically isolated mature embryos of *T. durum* var. PDW215 were inoculated with *Agrobacterium* [LBA 4404 (pCAMBIA3301:*PIN2*)] at $\sim 5 \times 10^8$–1×10^9 cells/ml density alongwith the phenolic inducer acetosyringone (200 μM). Washing with $MS_{1/2}$ was undertaken after 3 days of co-cultivation on MSE2As (MSE2 + 200 μM, acetosyringone) and then histochemically assayed for GUS expression. Callusing was induced on $MSE2P_5C_{250}$ [MSE2 medium with phosphinothricin (5 μg/ml) selection and a bacteriostatic agent-cefotaxime (250 mg/l)] for 3 weeks thereafter which the calli were

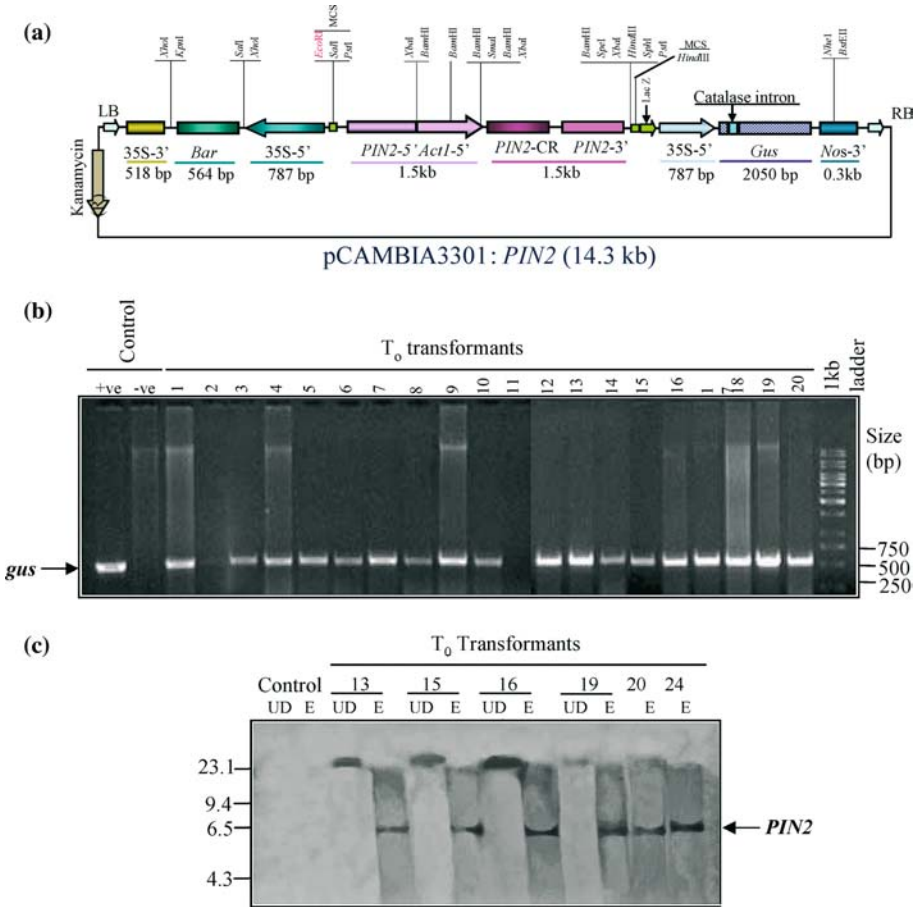

Figure 1. *Agrobacterium*-mediated transformation of mature embryos of *Triticum durum* var. PDW215 (a) Schematic map of the vector pCAMBIA3301:*PIN2* (14.3 kb).(b). PCR analysis of T0 transformants using primers specific to *Gus* gene. The plasmid pCAMBIA3301:*PIN2* and genomic DNA from an untransformed plant were used as positive and negative control, respectively. Numerals on the right indicate size of the DNA fragments of the length standard in bp (1kb Ladder DNA).(c) Southern analysis of T0 transformants. Lane 1 and 2, untransformed control [undigested (UD) and digested with *Eco*RI (E), respectively], Lanes 3–12, genomic DNA of putative transformants digested with *Eco*RI. Hybridization was performed with the 3 kb *PstI* fragment of pCAMBIA3301:*pin2* that spans the *PIN2* coding region.

transferred to MSER (2.22 μM BAP and 0.1 μM NAA) for another 2–3 weeks alongwith phosphinothricin (1.25 μg/ml) selection. The plantlets thus obtained were transferred to $MS_{1/2}$ + NAA (0.1 mg/l) for rooting. The rooted plantlets were transferred to earthen pots containing a mixture of soilrite (Kel Perlite, Bangalore, India) and soil (1:1) and grown to maturity in a growth chamber (Conviron, Control Environments Limited, Winnipeg, Canada) operating at 21°C, 16 h light at 100–125 μmol/m^2/s and 70% relative humidity. The plants were supplied with a liquid medium recommended for growth of wheat plantlets (Lee et al., 1989).

Transgene detection by PCR

Screening of the putative transformants was undertaken by PCR analysis (Mullis & Faloona, 1987). Primers for *Bar*, *gusA* and *pinII*, gene were designed by using the programme Gene Runner™ software (npt5 5′TCGGCTATGACTGGGCACAACAGA3″, npt3 5′AAGAAGGCGATAGAAGGCGATGCG3′, bar5 5′ACCATCGTCAACCACTACATCG3′, bar3 5′TCTTGAAGCCCTGTGCCT3′, bar3 5′TCTTGAAGCCCTGTGCCTC3′, gus5 5′CTCGTCCGTCCTGTAGAAACCC3′, gus3 5′CAGGTGTTCGGCGTGGTGTAG3′, pin5 5′ATGGCTGTTCACAAGGAAGTT3′, pin3

5′TCACATTGCAGGGTACATATTTG3′). PCR amplification was performed as per manufacturer's instructions (MBI Fermentas, USA), by initial denaturation at 94°C (5 min hold), followed by 25–30 cycles at 94°C (30 s), annealing (30 s) and extension at 70–72°C (30 s) with a final holding at 72°C (7 min) for extension employing a Perkin-Elmer Gene Amp PCR system 2400 or Gene Amp PCR system 9700.

The putative transformants (T_0) evaluated by PCR amplification for the presence of *PIN2*, *Bar* and the *gusA* genes were further verified for stable integration by Southern analysis. The T_1 progeny were further assessed for the stable integration and segregation *via* PCR assay and the χ^2 analysis undertaken for investigating the segregation pattern. The progeny of line 7 (T_1 generation 7.1, 7.2, 7.3) were analysed for the *Bar* gene expression also and the χ^2 analysis of the T_1 progeny was appraised to study the segregation ratio. The nematode infestation was undertaken in the progeny of the lines 7.1a–l, 7.2a–s and 7.3a–u.

Southern analysis of putative transformants

Genomic DNA (10–20 μg) of putative transformant lines as well as the progeny lines was digested overnight at 37°C with 50–60 units of appropriate restriction enzyme. The prepared Southern blot was incubated in prehybridization solution overnight at 37°C with shaking at 40 rpm. The probes for *PIN2*, *Bar* as well as the *gusA* genes were used after digesting the vector (pCAMBIA3301:*PIN2*) with relevant enzymes and resolving in 1% LMP gel.

Assay for Bar gene expression

Phosphinothricin leaf paint assay. The progeny of transgenic plants with Bar gene as the selectable marker were analysed by phosphinothricin leaf paint assay. Leaf painting was executed as described by Lonsdale et al. (1998) and PPT resistance was ascertained according to the percentage of necrosis suffered by the leaf.

Modified CR enzyme assay. To validate the phosphinothricin leaf paint assay results, the modified chlorophenol red (CR) assay was also carried out (Kramer et al., 1993) on the transformed lines. Non-transformed shoots incubated under the same conditions were used as a control. A colour shift from red to orange/yellow was considered as a 'resistant' reaction. A change to purple shade indicated the absence of *Bar* gene expression concomitant with the accumulation of ammonia.

GUS fluorimetric assay

The reporter gene activity was histochemically localized in the explants according to the protocol described by Jefferson et al. (1987). The relative fluorescence was measured by employing Hoefer (DyNA quant 200, Amersham International Inc, UK) fluorometer at an excitation wavelength of 365 nm and emission wavelength of 455 nm.

Proteinase inhibitor assay

The biotic assay involved infecting 10–15 day-old wheat seedlings with freshly hatched second stage juveniles of *H. avenae*. About 4000 juveniles were inoculated per plant over a period of 3–4 weeks. Since homozygous dominant plants could not be identified in the lines transformed with pCAMBIA:*PIN2*, therefore progeny of the lines 7.1, 7.2 and 7.3 (7.1a–l; 7.2a–s; 7.3a–u) were used for nematode infestation and subsequent analyses of PIN2 expression levels. Untransformed PDW215 seedlings served as the negative control plants. The plants were grown and maintained in a growth chamber as described earlier.

The systemic expression of PIN2 protein triggered in the transformants due to *H. avenae* infestation was measured based on proteinase inhibitory (PI) activity in the leaf extracts against commercial bovine pancreatic chymotrypsin (Duan et al., 1996) using ATEE (N-acetyl-L-tyrosine ethyl ester) as the substrate, as per the manufacturer's instruction. Commercially available chymotrypsin inhibitor II of potato (Calbiochem) served as a positive control towards the plot of the standard curve while protein extracts of non-transfomed wheat plant served as negative control. The PIN2 expression levels were correlated with the phenotypic changes observed in the T_2 generation.

Plant phenotypic changes incurred due to infestation

Evaluation of phenotypic changes was observed in the plants at the mature plantlet stage. The plant

height was measurement in cm, while the mature caryopses were allowed to dry and then harvested and the total number of seeds per plant and the seed weight were determined. The correlation and regression analysis was undertaken at World Wide Web: http://www.stattucino.com/to score the impact of PI levels in conferring nematode resistance.

Results and discussion

During the present investigation potato proteinase inhibitor (PIN2) gene was introduced into wheat (*T. durum*, PDW215) via *Agrobacterium*-mediated mature embryo co-cultivation approach with an aim to analyse its integration and subsequent expression pattern.

Generation of pin2 *transgenic plants*

Although most regeneration studies in wheat have been confined to immature embryos, scutellum and also to immature inflorescence tissue (Patnaik & Khurana, 2001), nonetheless, mature embryos have also been employed for callusing, regeneration and transformation studies (Mahalakshmi et al., 2000; Patnaik & Khurana, 2001; Khurana et al., 2002). Ozgen et al. (1996a, b) appraised the callusing response of mature and immature embryos of seven genotypes of winter *durum* wheat cultured on MS medium supplemented with 2,4-D and found that mature embryos had low frequency of callus formation but a high regeneration capacity in comparison with immature embryos. Moreover, the availability of mature wheat seeds throughout the year provides a ready source of explant and hence mature embryos were employed as primary explants for the induction of embryogenic callus after excising aseptically from the caryopses. Monocotyledonous plants including important cereals were earlier thought to be recalcitrant to *Agrobacterium*-mediated gene transfer but the scenario has changed in the last few years with the report of stable *Agrobacterium*-mediated transformation events in wheat (Cheng et al., 1997; Hu et al., 2003; Khanna & Daggard, 2003; Wu et al., 2003). Nonetheless, variations in transformation frequencies are frequently reported and influenced by differences in the genotypes (Takumi & Shimada, 1996), physiological status of the donor plant and also the mode of transformation procedure adopted (Pellegrineschi et al., 2002). Although, *durum* wheat transformation has been accomplished by the biolistics approach (*see* Patnaik & Khurana, 2003), in the present study, a novel *Agrobacterium*-mediated transformation protocol using the mature embryos has been employed successfully (Patnaik et al., 2005).

Agrobacterium-mediated [LBA4404 (pCAMBIA3301:*PIN2*)] gene transfer efficiency was evaluated by analysing the transient expression of the transgenes such as the *gusA* gene in the explants following three days of co-cultivation as well as evaluating stable transgene expression at the plantlet stage. Based on Southern analysis the transformation efficiency was 3%, while the frequency of escapes was 35.71%. The T_0 putative transformants were analysed for the stable integration and expression of the respective genes and the positive lines analysed further for stability and expression levels. The phenotypic appearance of the seeds obtained from the T_0 lines showed remarkable differences and also revealed variable germination percentage (Table 1).

Screening of transformants for gusA *and* Bar *gene expression*

The various methodologies available to study the integration, segregation and expression pattern of the transgenes were tested to conclusively prove the efficacy of gene expression in the transformants. To confirm the presence of transgenes in the primary T_0 transformants, PCR amplification of genomic DNA was undertaken using primers specific to *gusA* (Figure 1(b)), *Bar* and the *PIN2* genes. No amplified product was detected in the samples containing genomic DNA from an untransformed plant. Southern analysis highlights the successful integration of the T-DNA in the T_0 transformants when the *gusA* as well as *pin2* (Figure 1(c)) genes were used as probes. The T_1 transformants were also evaluated to analyse the segregation pattern of the *gusA*, *Bar* and *the pin2* genes. The χ^2 analysis of the T_1 progeny based on the *Bar* gene expression show permissible χ^2 values (for 3:1 ratio segregation) falling within the table value at 1 degree of freedom (Table 2). The *gusA* positive segregating plantlets were identified by the presence of a PCR amplified *gusA* product (549 bp) and the χ^2 analysis of the T_1 segregating progeny lines was also evaluated (Table 3). A

Table 1. Summary of T_1 *T. durum* var. PDW215 transformed with pCAMBIA3301:PIN2

S.No	Parental line (T_0)	Total No. seeds obtained (T_1)	Shriveled seeds	Appearance of seeds	Germinated seeds transferred to pots	% Germination
1	3	9	9	All seeds shriveled	5	55.55
2	4	17	9	Opaque and translucent	9	52.94
3	6	3		Translucent	3	100.00
4	7	18	2	3 yellow, opaque; others translucent	14	77.77
5	8	8		Translucent	6	75.00
6	9	17		Shriveled and opaque	14	82.35
7	12	–				
8	13	30		Opaque and yellow	12	40.00
9	15	10	2	Translucent	6	60.00
10	17	13		Translucent and opaque	–	–
11	19	46	3	Opaque and translucent	–	–
12	20	14	2	Translucent	11	78.57
13	24	22	2	Opaque and yellow	17	77.27

Table 2. χ^2 analysis of T_1 progeny based on *bar* gene expression

S.No	Parental line (T_0)	T_1 line (number of progeny lines)	Observed (+ve)	χ^2 (3:1)	*p*-value
1	4	9	8	0.926	0.335
2	6	3	2	0.110	0.738
3	7	14	12	0.857	0.354
4	9	14	13	2.381	0.122
5	13	12	10	0.444	0.504
6	15	3	2	0.111	0.738
7	17	2	2	0.667	0.414
8	20	4	3	0.000	1.000
9	24	7	7	2.333	0.126

Table 3. χ^2 analysis of progeny lines

Parental line	Total No. seeds	No. of progeny lines analysed	Observed (+ve)	Assayed for	χ^2 (3:1)	*p*-value
T_0 line						
7	14	12	10	*gus*	0.444	0.504
7	14	12	12	*bar*	0.857	0.354
T_1 line						
7.1	12	12	9	*bar*	0.000	1.000
7.2	14	9	9	*bar*	3.000	0.083
7.3	22	16	11	*bar*	0.291	0.589

*Table value is 3.84 (for 1 degree of freedom) at 0.05 level of significance.

similar trend was observed in the χ^2 analysis of the T_2 segregating lines 7.1a–l; 7.2a–s; 7.3a–u (progenies obtained from 7, 9 and 24 lines). Though most plants segregate with 3:1 ratio, exceptions were noticed as in the case of 9 progeny plants, where the χ^2 value is greater than the table value implicating alternate genetic segregation. The fluorescent MUG assay was more sensitive than the histochemical X-GLU assay and the GUS expression levels in the untransformed control was in the order 10 ± 0.75 MU/μg protein/min. In the T_2 generation line 7.1a–l, 7.2a–s (Figure 2(a)) and 7.3a–u the GUS specific activity varied co-relating probably with the segregation of the gene.

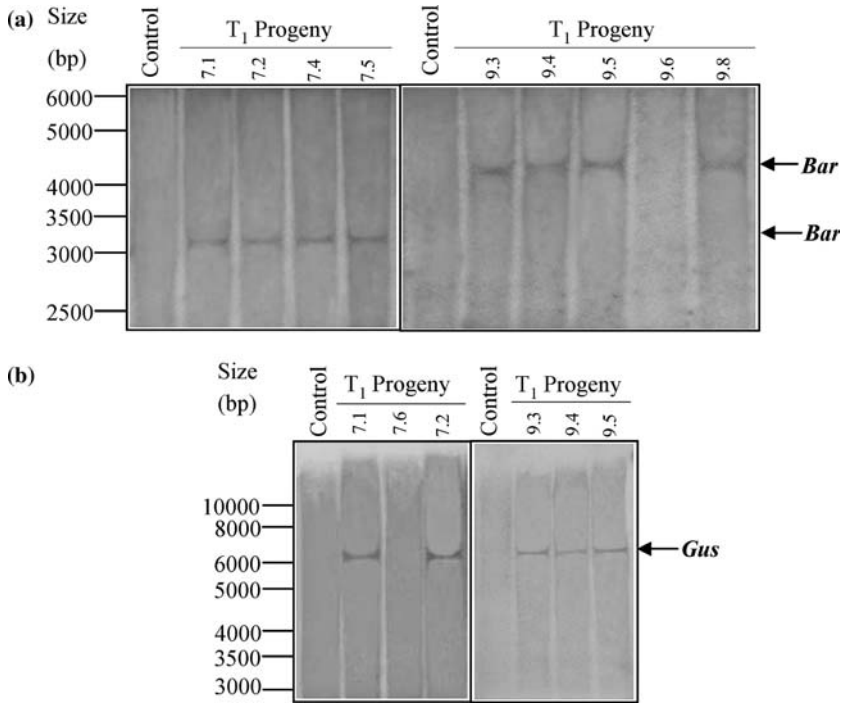

Figure 2. Southern analysis of T1 transformants digested with *Eco*RI. Numerals on the left indicate size of the DNA fragments of the length standard in kb/bp (1 kb Ladder DNA/λ Hind III DNA). (a) Hybridization performed by *Xho*I fragment of pCAMBIA3301 spanning the *Bar* coding region. (b) Hybridization performed by *Nco*I and *Bst*EII fragment of pCAMBIA3301 spanning the *gusA* coding region.

However, in the present study, the transgenic lines obtained *via Agrobacterium*-mediated transformation events with *gusA* as a reporter gene displayed low GUS activity that was undetectable by histochemical assay. Similar results have also been reported by Nehra et al. (1994) and Srivastava et al. (1999).

The *Bar* selection in wheat has been investigated by numerous researchers in both *T. aestivum* as well as in *T. durum* (Patnaik & Khurana, 2001). In the present study the *Bar* gene segregation and expression was evaluated by undertaking PCR analysis followed by chlorophenol red assay along with the phosphinothricin leaf paint assay (data not presented) suggesting stable integration, segregation as well as the expression of the selectable marker in both the T_1 (Tables 2 and 3) as well as the T_2 lines. The χ^2 analysis of the T_1 lines based on the *Bar* gene PCR highlights stable integration as well as segregation in the expected 3:1 ratio (Table 2 and 3) at $p \leq 0.05$ level of significance. Most *durum* wheat transformation so far reported have also been undertaken using *Bar* as a selectable marker. The plants detected PCR positive for the *Bar* gene (295 bp) suffered little or no damage upon leaf paint assay with phosphinothricin thus demonstrating the functional activity of the *Bar* gene, whereas the leaves of untransformed control and few putative transformants developed yellow spots. Distinct integration patterns were observed in the T_1 transformants when the selectable marker gene, *Bar* (left panel) as well as the scorable marker gene, *gusA* (right panel) were used as probes for Southern analysis (Figure 2). The progeny line 7 (~3.1 kb) differed with respect to the progeny line 9 (~4.4 kb) when *Bar* was employed as a probe (Figure 2(a)). However, Southern analysis with the *gusA* gene shows an analogous profile (~6.5 kb, Figure 2(b)) similar to the parental lines.

Analysis of PIN2*-putative transformants*

The nematode inoculation was given at 10-day-old-seedling stage thereafter which the PIN2 protein levels were assessed in the mature plants. The integration, segregation and expression pattern of the *PIN2* gene was evaluated along

with the *Bar* and *gusA* genes. Since homozygous *PIN2* dominant plants could not be identified, therefore arbitrarily the progeny of 7.1, 7.2 and 7.3 lines (7.1a–l; 7.2a–s and 7.3a–u) were grown in clay pots and analysed after nematode infestation. Noteworthy phenotypic changes could be observed in the T_2 generation (7.1a–l; 7.2a–s and 7.3a–u) after subsequent nematode infection (Figure 3). The systemic expression of the *PIN2* gene elucidates effective transduction of wounding signal efficiently from root to leaves. The susceptible untransformed control plants showed stunted height as did the non-expressing lines, while the transformants with induced *PIN2* expression showed enhanced growth probably due to reduced occurrence of nematode infestation in the roots. However, for the complete picture the *in vivo* studies highlighting the progress of the nematode infestation would prove beneficial.

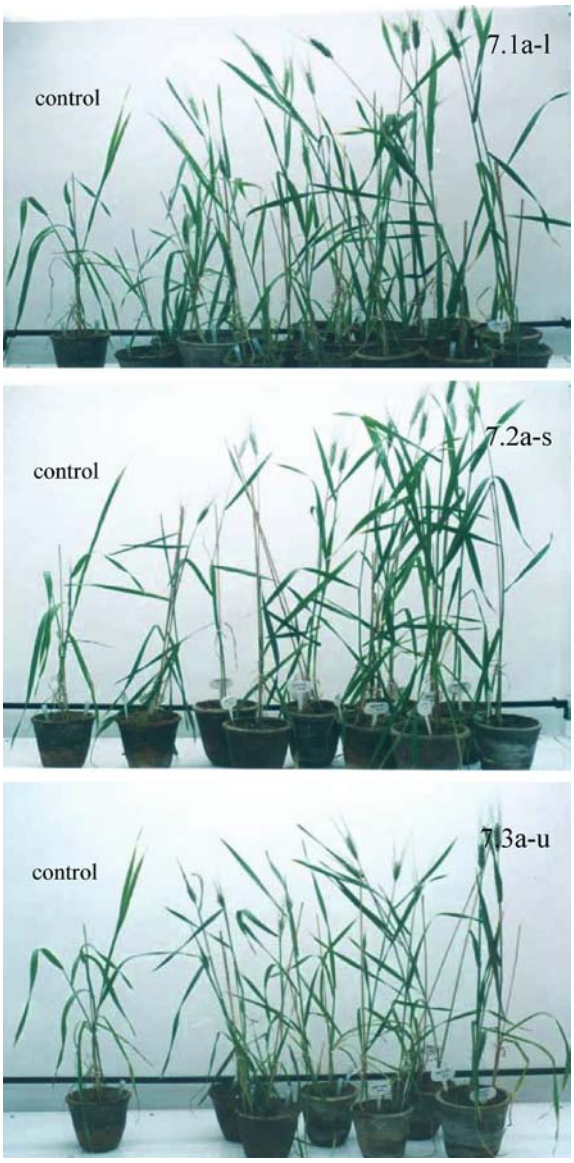

Figure 3. Phenotypic changes observed in the T2 generation (7.1a–l, 7.2a–s and 7.3a–u progeny lines) after nematode infection.

Proteinase inhibitor (PI) assay was undertaken using the substrate ATEE to evaluate the ability of the crude plant protein extract to inhibit chymotrypsin. The data are expressed relative to the control (absence of transgenic total plant protein abstract/potato chymotrypsin inhibitor II, Calbiochem) reveals the relative PI values (Figure 4(a)). The relative PI value of the negative control (untransformed plant) was 0.151 ± 0.02. The relative PI values were observed to correlate significantly with the plant height suggesting the direct influence of PI in conferring nematode resistance in the progeny lines (Figure 5). A few plants that might have escaped infestation were found to differ from the observations and thus were omitted from the correlation and regression analysis plot. Thus it can be concluded that PIN2 systemic expression confers satisfactory nematode resistance and therefore the transformants are phenotypically comparable to untransformed control in having similar height. The correlation and regression analysis suggests that at $p \leq 0.05$ level of significance the regression values of most parameters analysed (relative PI values vis-à-vis plant height, plant seed weight and number of caryopses) were observed to be significant (Table 4). A positive correlation (R^2 values were observed to fall within the table value at $p \leq 0.05$ level of significance) was observed between the plant height and the relative PI values of the lines 7.1a–l, 7.2a–s and 7.3a–u (Figure 5). The slope of the regression line highlights a positive correlation between most variables analysed, while the coefficient of determination (R^2, given alongside the figures) also indicates a positive strength of the correlation (Figure 5). Moreover, it was also observed that higher PI values positively influenced plant productivity. For example seed weight as well as seed number of lines 7.2a–s consistently correlated with the relative PI values as shown by the Pearson's

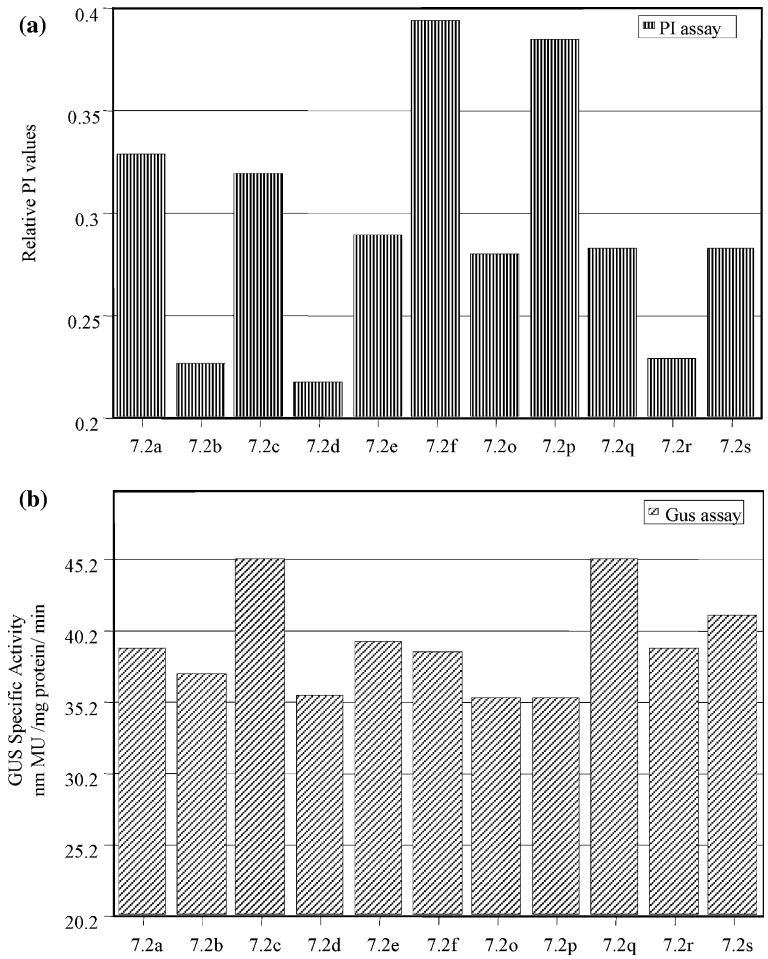

Figure 4. Relative PI values and GUS fluorometric analysis of line 7.2a–s. (a) Relative PI (Proteinase inhibitor) values of line 7.2a–s. (b) GUS fluorometric analysis of line 7.2a–s.

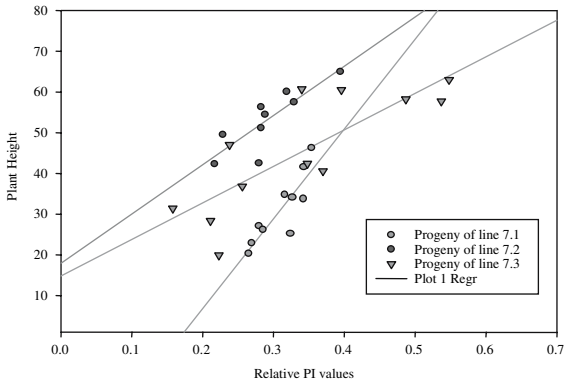

Figure 5. Correlation and regression analysis between plant height and the PI values analysed in selected representative lines (7.1a–l, 7.2a–s and 7.3a–u).

correlations value (Table 5). The correlation coefficient values of most progeny lines are well within the significant range ($p \leq 0.05$ level) and are therefore statistically acceptable, though in Table 3a few progeny lines have been omitted from the regression plot analysis. For example, two progeny lines 7.2b and 7.2p have been omitted from regression plot analysis of the line 7.2a–s since their PI values were significantly higher than the correlation coefficient table value at $p \leq 0.05$ level and (8 degrees of freedom). However, the kinetics of induction of *PIN2* gene and the stability of the PIN2 protein produced in transgenic wheat plants needs to be deciphered further so as to understand the resistance mechanisms at play. It would also be interesting to see the effect, if any, of the PIN2 production on growth and development

Table 4. Summary of parameters analysed in few selected plants of line 7.2a–s

Progeny	PI value	Plant ht (cm)	Seed No.	Seed wt.
7.2a	0.3292	57.50	27	0.7732
7.2c	0.3194	60.10	13	0.6902
7.2d	0.2172	42.25	4	0.1966
7.2e	0.2890	54.50	8	0.3618
7.2f	0.3946	65.00	17	0.7330
7.2o	0.2798	42.50	0	0.0000
7.2q	0.2828	51.20	11	0.3526
7.2r	0.2288	49.50	10	0.3466
7.2s	0.2828	56.20	10	0.4709

The table value of correlation coefficient at $p \leq 0.05$ level and (8 degrees of freedom) is 0.632.
The progeny lines 7.2b and 7.2p have been omitted from regression plot analysis.

Table 5. Pearson Correlation analysis in few selected plants of line 7.2 calculated by Statticino.com

	PI value	Plant height	Nos. of seeds	Seed wt.
Pivalue	1.0			
Plant ht.	0.83979	1.0		
Seed no.	0.61115	0.72925	1.0	
Seed wt.	0.72322	0.9158	0.90316	1.0

of *H. avenae* juveniles in the various transgenic lines. This, however, remains to be accomplished in the future. Nonetheless, the present study successfully demonstrates (i) the potential use of *A. tumefaciens* for transformation of *T. durum* mature embryos as explants, and (ii) nematode resistance conferred by the use of a serine proteinase inhibitor (*PIN2*) gene.

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