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Screening of *Agrobacterium tumefaciens* strains for wheat *(Triticum aestivum)* transformation

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ABSTRACT

Five *Agrobacterium tumefaciens* strains with 'GUS' reporter gene and 'selectable marker genes' for Kanamycin (for *Agrobacterium*) and Hygromycin (for plant) were co cultivated with immature embryos of regeneration responsive wheat genotypes on N_6 medium supplemented with different concentrations of 'acetosyringone'. The immature embryos of variety HD 2329, co-cultivated on 40 mgl^{-1} acetosyringon developed agroinfection of two *A. tumefaciens* strains, EHA 105 and LBA 4404 when subjected to 'GUS assay and developed blue spots. The expression of LBA 4404 was found to be more virulent over EHA 105 strain. Growth of bacterial cells was arrested with Cefotaxime (150mgl^{-1}) without affecting the growth of callus.

Key Words: *Triticum aestivum*, Wheat Transformation, *Agrobacterium tumifaciens*, Agroinfection, Transgenic.

INTRODUCTION

Wheat is the major cereal crop next to rice and is the major source of energy for the world population. Among different wheat species, *T. aestivum*, the bread wheat (hexaploid) has major share of cultivation and *T. durum* or durum wheat (tetraploid) is the second species under cultivation. However, *T. diococcum* is also cultivated in some parts of the world. The conventional breeding techniques have paid its dividends by increasing wheat production of India from 9.5 million tons in 1963-64 to 74.37 million tons in 1999-2000. (Nagarajan 2000). However, the attempts to develop hybrids in breaking further yield barriers in wheat have gone futile.

In the present scenario, development in the field of wheat biotechnological approaches is showing promise in introducing desired novel genes available in alien sources to broaden the genetic base for various biotic and abiotic stresses and increasing the input-use efficiency (Pingali and Rajaram 1999). Under the WTO regime, the global wheat industry is undergoing a transformation and there is need to concentrate Indian wheat research efforts in the direction of quality improvement for meeting the prescribed wheat quality standard of the world market. Quality traits are generally governed by a single or few genes, are very difficult to handle through conventional breeding due to inflow of associated deleterious traits. In such situations, development of transgenic plant appears to be a better option. Introducing the *barnase-barstar* gene using transformation techniques can impart perfect genetic male sterility and fertility restoration to a hybrid programme (De Block *et al.* 1997).

Among various methods available for transformation, the biolistic-gun approach was considered to be the most successful method in delivering foreign genes into intact plant tissues of wheat by particle bombardment and revolutionized the field of plant transformation (Klein *et al.* 1987; Klein and Jones 1999). Later, agroinfection of soil bacterium was reported in monocot plants (Dale *et al.* 1993). *Agrobacterium tumefaciens* mediated transformation (Hieti *et al.* 1995 and Vain *et al.* 1995), however, non-specific to cereal crops, is a natural phenomenon and has advantages of better gene integration and expression over 'Particle Bombardment' (Klein *et al.* 1987 and Dale *et al.* 1993). Moreover, the *Agrobacterium* mediated approach is less expensive. The present studies were carried out in identifying the virulent strains of *Agrobacterium* to Indian genotypes for standardization of transformation procedure for wheat.

Table-1: A list of important resistant genes transferred to wheat from alien sources.

Gene	Source
Resistance to Leaf rust	
Lr9	*Aegilops umbellulata*
Lr18	*Triticum timopheevi*
Lr19	*Thinopyrum*
Lr23	*T. turgidum*
Lr24	*Ag. elongatum*
Lr25	*Secale cereale*
Lr29	*Ag. elongatum*
Lr32	*T. tauschii*
Resistance to stem rust	
Sr2	*T. turgidum*
Sr22	*Triticum monococcum*
Sr36	*Triticum timopheevii*
Resistance to stripe rust	
Yr15	*Triticum dicoccoides*
Resistance to powdery mildew	
Pm12	*Aegilops speltoides*
Pm21	*Haynaldia villosa*
Pm25	*T. monococcum*

Resistance to wheat streak mosaic virus

Wsm1 *Ag. elongatum*

Hessian fly

H21 *Secale cereale*
H23 *Secale cereale*
H24 *T. tauschii*
H27 *Aegilops ventricosa*

Cereal cyst nematode

Cre3 *T. tauschii*

MATERIAL AND METHODS

Maintenance of Agrobacterium tumefaciens strains: Following purified strains of *Agrobacterium tumefaciens* having ß-glucuronidase (GUS) reporter gene with 'selectable marker genes for kanamycine (for bacterium selection) and hygromycine (for plant selection) were procured from Indian Agricultural Research Institute, New Delhi and Punjab Agricultural University Ludhiana were maintained saperately on liquid bacterial culture mediums (A281, ALG1, EHA 101, EHA 105 on 'YEM' Rif 10 Kan 50 and LBA 4404 on 'YP' Kan 50 Hyg 50) by preparing their suspension culture in incubator shaker at 28°C over night. The incubator was rotated at 250 rpm. The bacterial plating was done by putting their streak on solidified bacterial culture medium (Fig.1&2). The resistant colonies developed on screening media were collected for their co cultivation with immature embryos of wheat varieties.

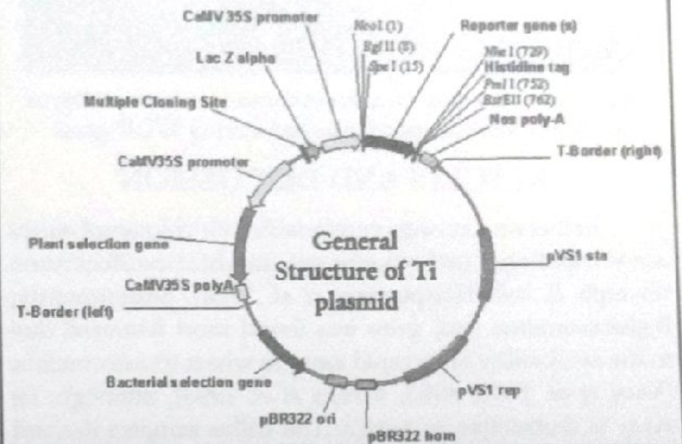
A281 : (succinamopine, leucinopine, agrocinopine type) reconstructed strain, derivative of A136 (cured C58) harbouring pTiBo542, super-virulent.

AGL1: Its virulence is weaker than A281.

EHA101 :(leucinopine, succinamopine, agropine type) genotype is AGL0 (C58 pTiBo542), Km(R); super-virulent.

EHA 105 : Km(S) derivative of EHA101.

LBA 4404 : (agrocinopine, octopine type) (Ach5 pTiAch5) Sm/Sp(R) in the virulence plasmid (from Tn904).



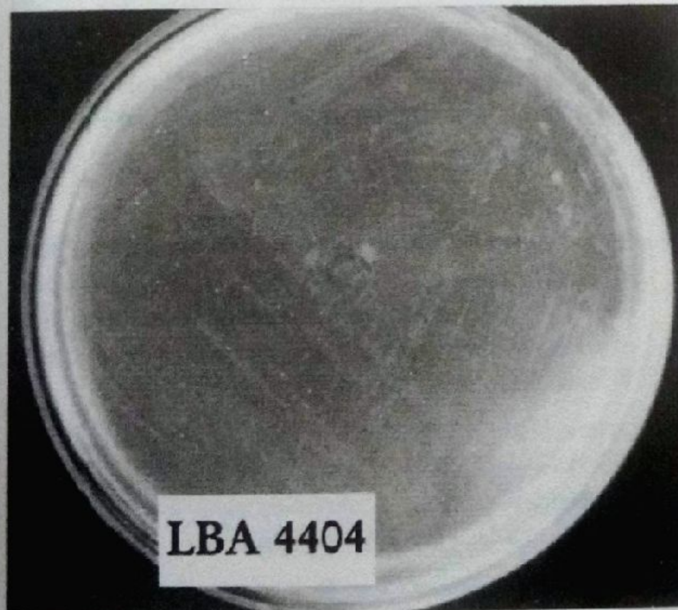


Fig. 1: Maintenance of LBA 4404 *Agrobacterium tumefaciens* colonies on YP medium.

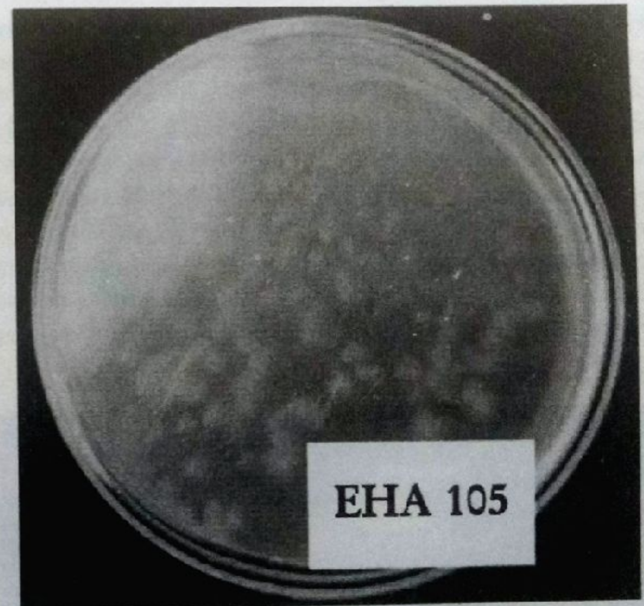


Fig. 2: Maintenance of EHA 105 *Agrobacterium* colonies on YEM medium.

Wheat Transformation: The immature embryos (11-13 day old) excised aseptically from field grown plants of Indian wheat genotypes namely PBW 343, HD 2329, HI 1077, HP 1731, HUW 234, UP 2338, UP 2425 and WH 542 were cultured on MS based callus induction media for 3-5 days. The single bacterial colonies were re-suspended in N_6 based liquid medium having different concentrations (40, 100 and 150 mg l^{-1}) of 'acetosyringone' as exogenous opine source for enhancing agroinfection. 3-5 days old callus derived from immature embryos was co-cultivated with *Agrobacterium* for five minutes (Fig.-3). The filter-dried callus was transferred on N_6 callus induction medium having 'acetosyringone' and incubated in dark for 2-3 days at 24°C. Callus was further cultured on MS medium having different concentrations (100, 150 and 200mg l^{-1}) of 'cefotaxime' for *Agrobacterium* free embryogenic callus growth. The callus samples were taken and subjected to 'gus assay' with X-gluc solution. The remaining callus was transferred on hygromycine resistant screening medium.

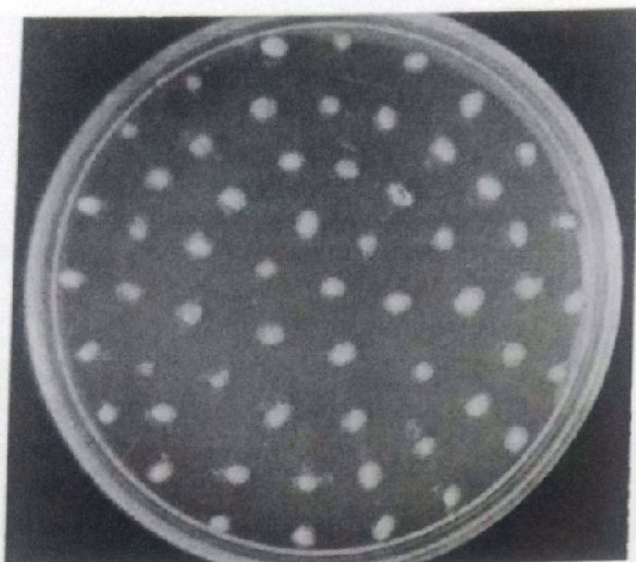


Fig. 3: Co-cultivation of callus induced immature embryos with *Agrobacterium tumefaciens* harbouring 'GUS' gene.

RESULTS AND DISCUSSION

Earlier workers were comfortable with 'chloramphenicol acetyl transferase' (*cat*) reporter gene in wheat transformation through *E. coli* (Hauptmann *et al.* 1988). Subsequently, ß-glucuronidase (*gus*) gene was found most favoured due to the availability of its rapid assay in wheat transformation (Vasil *et al.* 1992, 1993, Weeks *et al.* 1993), although, its assay is destructive in nature. The callus samples derived from immature embryos when exposed to 'GUS Assay' developed blue spots only for variety HD 2329 which were co-cultivated with two *Agrobacterium* strains i.e. EHA 105 and LBA 4404. This indicated that both EHA 105 and LBA 4404 has vorulant reaction for wheat variety HD 2329. Although, the *Agrobacterium* strains EHA 105 and LBA 4404 were found to be virulent, but the expression of LBA 4404 was found to be more virulent over EHA 105 strain (Fig.4). Now, the non-destructive reporter genes encoding for anthocyanin biosynthesis, green fluorescent protein, and firefly luciferase are also available to conclude the results of transferred genes in living cells.

Fig. 4: Transient expression of 'gus gene' on immature embryos of HD 2329 variety co-cultivated with LBA 4404 *Agrobacterium tumefaciens* strain on MS based callus induction media containing 40 mgl^{-1} asetocyringon.

The confirmation of agroinfection only on N_6 callus induction media having 40 mg l^{-1} acetosyringone concentration indicated that 40 mg l^{-1} concentration of acetosyringone was optimum enhancing the infection of *Agrobacterium*.

The bacterial cell continued to grow on lower concentration (100mg l^{-1}) of cefotaxime but the growth of bacterial cells was arrested with cefotaxime at 150mg l^{-1} concentration without affecting the growth of plant cells. However, and plant cells become dead at higher cefataxime concentration (200mgl^{-1}). Therefore, it is concluded that 150mg l^{-1} concentration of cefotaxime is optimum for getting rid of bacterium cells from callus cultured on transient expression screening media.

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