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AN IMPROVED *AGROBACTERIUM* MEDIATED TRANSFORMATION SYSTEM IN WHEAT

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Abstract

Wheat is the world's largest volume crop traded internationally. Foliar diseases are the main biotic restriction reducing yield in wheat crops. Wheat productivity can be enhanced through controlling foliar disease through the use of resistant cultivars. Plant breeders are providing considerable attention to wheat genetic improvement from the past few years to minimize losses due to pests and pathogens and also to improve the grain yield. Genetic transformation is fundamental to wheat molecular genetics and improvement through genetic engineering. There has been significant progress in *Agrobacterium* transformation of wheat but it is still confined mainly to a few responsive varieties due to poor understanding of the mechanism and availability of efficient techniques. This system is influenced by a number of factors including acetosyringone concentration. In present study, the pattern of interaction in between acetosyringone concentration and transformation potential was exploited for the efficient development of *Agrobacterium* mediated transformation system in local cultivars of wheat (Inqilab-91 and Chakwal-97). Mature embryos were used as explant sources for callus induction, which were used later in transformation experiments. Different concentrations of acetosyringone were used and it was observed in the study that transformation efficiency increases with increase in acetosyringone concentration upto 400 µM but further increase beyond this drastically reduced the transformation frequency. Maximum transformation efficiencies of 56.0 and 52.0% were obtained with 400µM of acetosyringone from Inqilab-91 and Chakwal-97 respectively.

Introduction

Wheat is the world's second largest crop, supplies 19% of human calories and is the largest volume crop traded internationally (Atchison *et al*., 2010). Foliar diseases are the main biotic restriction reducing yield in wheat crop affecting both, grain number and/or grain weight, depending on developmental stage at which infection occurs (Serrago *et al*., 2011). Wheat productivity can be enhanced through controlling foliar disease through the use of resistant cultivars (Berry *et al*., 2008). Plant breeders are providing considerable attention to wheat genetic improvement from the past few years to minimize losses due to pests and pathogens and also to improve the grain yield (Pingali & Rajaram, 1999; Hussain *et al*., 2010; Khan & Khan, 2010). Genetic transformation is fundamental to wheat molecular genetics and improvement through genetic engineering. *Agrobacterium*-mediated transformation and microparticle bombardment are the two widely used methods for wheat genetic transformation (Lazzeri & Jones, 2009; He *et al*., 2010).

There has been significant progress in *Agrobacterium* transformation of wheat but it is still confined mainly to a few responsive varieties with quite different transformation frequencies (Hu *et al*., 2003), due to poor understanding of the mechanism and availability of efficient techniques for selecting breeding resources for stress resistance (Bhatti & Chaozu, 2009). This system is influenced by a number of factors such as bacterial strains, plasmids, tissue culture environment, media for explant culture, co-cultivation duration, acetosyringone (AS), explant wounding, selective marker and vector and competency of target plant tissues for infection (Briza *et al*., 2008, Cho *et al*., 2008; Karami *et al*., 2009). The theme of this study was to exploit the pattern of interaction in between acetosyringone concentration and transformation potential for the efficient development of *Agrobacterium* mediated transformation system in local cultivars of wheat.

Materials and Methods

Embryos of wheat cv. Chakwal-97 and Inqilab-91 were used as explant source on MS (Murashige & Skoog, 1962) media supplemented with 3 mg/l 2,4-D for callus induction. The cultures were incubated in growth room for 22 days for proliferation. For further growth and proliferation, these calli were shifted to the same fresh medium after 22 days. Twenty two days old calli were used in the transformation experiments with *Agrobacterium* strain EHA101 harboring binary vector pIG121Hm (Fig). The calli were co-cultivated with the above said *Agrobacterium* for 1min and then were blotted dry. Co-cultivation was carried out with callus induction medium (CIM) + different concentrations of Acetosyringone i.e., 0.0, 50, 100, 150, 200, 300, 400 and 500µM. The plates were prepared for co-cultivation and after co-cultivation were placed in dark for 1-2 days at 28°C after the co-cultivation period, the calli were disinfected with 500 mg/l cefotaxime and were further proceeded for selection. The selection medium is also a MS media + 3 mg/l 2,4-D + 50 mg/l hygromycin and 500 mg/l cefotaxime. The disinfected calli were placed on the selection medium for 30 days. After thirty days, it was observed that some of the calli were turned brown or even black while some were still fresh and alive, these were the transformed calli and so were shifted to regeneration medium containing different growth regulators (2ip, IAA, BAP and Kn) and 50 mg/l hygromycin and 500 mg/l cefotaxime. Some calli were selected at random for GUS assay after 15 days of selection and were incubated in X-Gluc solution at 37°C for 2 days for confirmation of transformation.

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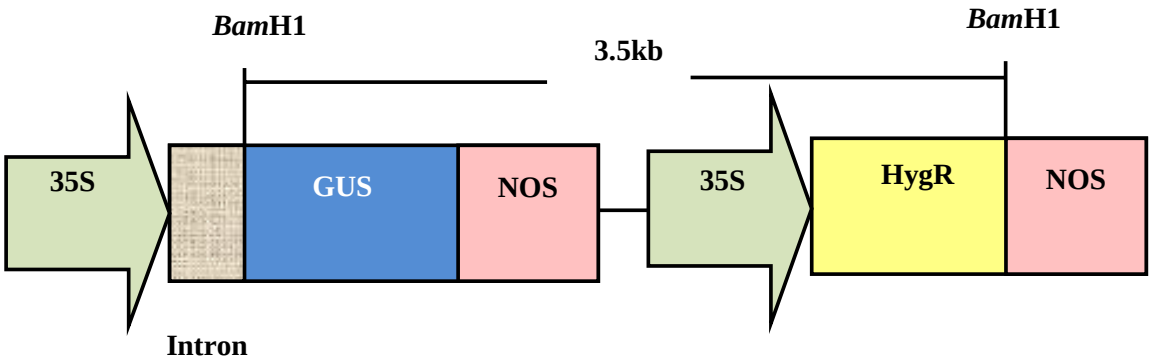

Fig. Partial Diagram of Binary Vector pIG121Hm.

Results and Discussion

Callus induction: Calli were induced from mature embryos of two wheat cultivars, Inqilab-91 and Chakwal-97 on MS medium with 3.0mg/l of 2,4-D along with a control without 2,4-D. This medium concentration was already optimized by Rashid *et al*., (2011). No callus induction was observed on the control medium in any of the five experiments, while highest average percentage of 70.14% was observed with Inqilab-91 (Table 1). Vendruscolo *et al*., (2008) achieved best callus induction on MS medium with 2.0mg/l of 2,4-D. According to Rahman *et al*., (2008), 6.0 mg/L of 2,4-D produced maximum percentage of calli (10.08%), while medium with lower concentrations of 2,4-D produced lower percentage of calli but larger size. These results are quite in contrast with our study. Nasircilar *et al*., (2006) achieved best callus induction results with MS medium supplemented with 2.0 mg/l of 2,4-D for different wheat cultivars. Haliloglu (2002) achieved best embryogenic callus induction from immature embryos of wheat on MS+B5 medium containing 2.0 mg/l of 2,4-D.

Table 1. Calli derived from mature embryos with 3.0mg/l 2,4-D.

S. No	Experiment No.	Inqilab 91		Chakwal 97	
		Control	3.0mg/l 2,4-D	Control	3.0mg/l 2,4-D
1.	Exp-1	0.0	63.4	0.0	56.9
2.	Exp-2	0.0	65.8	0.0	59.3
3.	Exp-3	0.0	66.2	0.0	65.8
4.	Exp-4	0.0	76.2	0.0	69.5
5.	Exp-5	0.0	79.1	0.0	70.8
Average		**0.0**	**70.14**	**0.0**	**64.46**

Regeneration of calli: Regeneration of the induced calli was carried out on MS medium containing 1mg/l BAP, 0.5mg/l Kinetin and 0.5mg/l 2iP. This regeneration medium combination was optimized by Rashid *et al*., (2011). Maximum regeneration of 75 % was observed from the mature embryos derived calli of Inqilab 91. Afzal *et al*., (2010) obtained maximum regeneration frequency of 46.66% on MS medium with 0.1mg/l of IAA and 0.5mg/l of BAP. Rahman *et al*., (2008) studied the effect of different combinations and concentrations of growth regulators on regeneration frequency of two spring wheat lines. They observed highest regeneration frequency of 13.63% on MS medium supplemented with 1.00 mg/L of kinetin. Alizadeh *et al*., (2004) found 1 mg/l of BAP, 0.2 mg/l IAA and 0.2 mg/l of 2,4-D as a best combination for shoot regeneration from embryos and 0.2 mg/l 2,4-D and 2 mg/l BAP as a best combination for shoot regeneration in excised embryo explants. Shah *et al*., (2003) found 2 mg/l BAP and 1.0 mg/l IAA as best combination for plantlet regeneration and also found Kn good regeneration. Malik *et al*., (2003) obtained maximum regeneration frequency of wheat on MS medium containing 0.5 mg/l of BAP and 0.1 mg/l of IAA. Shah *et al*., (2003), in their experiments obtained highest regeneration of wheat on MS medium containing 4.0 mg/l BAP alone or 2.0 mg/l BAP in combination with 1.0 mg /l IAA. Sarkar & Biswas (2002) achieved highest regeneration on MS medium containing 0.5 mg/l BAP and 0.5 mg/l kinetin. Rashid *et al*., (2002) obtained regeneration frequency on MS medium supplemented with 0.1 mg/l of IAA and 0.5 mg/l of BAP in wheat cv Rawal-87(Table 2).

Effect of acetosyringone concentration on transformation efficiency: Different concentrations of acetosyringone (0.0, 50, 100, 150, 200, 300, 400 and 500µM) were used at the time of co-cultivation and in the co-cultivation plates. Maximum transformation efficiencies of 56.0 and 52.0% were obtained with 400µM of acetosyringone from Inqilab-91 and Chakwal-97 respectively. No transformation was observed without the addition of acetosyringone. It was also observed that increase in As concentration from 50 µM upto 400 µM increased the transformation efficiency but further increase beyond 400 µM drastically reduced the transformation efficiency (Table 3). Ke *et al*., (2002) and McCormac *et al*., (1998) achieved best results with 100 µM As achieved best results by using 100µM As. Amoah *et al*., (2001) obtained highest percentage of explants producing blue spots with 200 µM of As.

Table 2. Regeneration frequency of mature embryos derived calli on regeneration medium with 1 mg/l BAP, 0.5mg/l Kinetin and 0.5mg/l 2iP.

Exp. No.	Total no. of explants		Plantlet formation		Regeneration frequency	
	T1	T2	T1	T2	T1	T2
1.	40	40	28	26	70.0	65.0
2.	40	40	28	27	70.0	67.5
3.	40	40	30	29	75.0	72.5
4.	40	40	31	29	77.5	72.5
5.	40	40	34	31	85.0	77.5
Average	**40**	**40**	**30.2**	**28.4**	**75.5**	**71.0**

T1 = Inqilab 91; T2 = Chakwal 97

Table 3. Transformation efficiency of two wheat cultivars cv. Chakwal-97 and Inqilab-91 using mature embryos as explant source with different concentrations of acetosyringone.

AC	TNE	CSGA	GPC	PGA	TCSH	SEH	TE
Chakwal -97							
00	50	25	1	4.0	25	0	0.0
50	50	25	7	28.0	25	8	32.00
100	50	25	8	32.0	25	9	36.0
150	50	25	10	40.0	25	10	40.0
200	50	25	13	52.0	25	12	48.0
300	50	25	14	56.0	25	13	52.0
400	50	25	16	64.0	25	14	56.0
500	50	25	9	36	25	6	24.0
Inqilab-91							
00	50	25	0	0.0	25	0	0.0
50	50	25	6	24.0	25	7	28.0
100	50	25	7	28.0	25	8	32.0
150	50	25	8	32.0	25	8	32.0
200	50	25	10	40.0	25	9	36.0
300	50	25	12	48.0	25	11	44.0
400	50	25	14	56.0	25	13	52.0
500	50	25	5	20.0	25	4	16.0

AC = Acetosyringone concentration in Mm, TNE = Total number of explants, CSGA = Calli selected for GUS activity, PGA = Percentage of GUS analysis, SEH = Selected explants on hygromycin, GPC = GUS positive calli, TCSH = Total calli for selection on hygromycin and TE = Transformation efficiency (%)

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