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Rapid *in-vitro* Propagation of Tomato (*Solanum lycopersicum*) *via* Cotyledonary Leaf Explants

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ABSTRACT

Tomato (*Solanum lycopersicum* L.) cv. PKM-1 cotyledonary leaf explants were cultured on MS basal medium supplemented with various concentrations of BAP, Kinetin, Zeatin, IAA and IBA. Among the various plant growth regulators combinations tried the best shoot regeneration was obtained when MS medium was supplemented with BAP 1.5 mg/L + Kinetin 1.0 mg/L and the best root regeneration was obtained when MS medium was supplemented with Kinetin 1.0 mg/L. Higher plantlet survival (86%) was obtained in soilrite mixture and 9.6 days has been taken for acclimatization.

Key words : BAP, IAA,Tomato, *in-vitro* cultures, Kinetin and Zeatin.

Tomato (*Solanum lycopersicum* L. 2n=24) is one of the most important vegetable crop and known as "protective food" because of its special nutritive value. Tomato is a rich source of minerals, vitamins and organic acids (healthy acids). In India, it is cultivated in an area of 5.20 lakh ha with a production and productivity of 90.06 lakh tonnes and 17,800 kg/ha respectively. In Andhra Pradesh, it accounts for 0.765 lakh ha with a production and productivity of 14.3 lakh tonnes and 19,000 kg/ha, respectively. Though, tomato has been subjected to genetic improvement using classical breeding methods for many years, full extent of exploitation of this crop has not been achieved as it is succumbed to several biotic and abiotic factors. Among several factors, the two important factors which limit the progress of breeding efforts are the availability of source of interest in sexually related plants and the duration of the reproductive cycle. The wild relatives of cultivated tomato especially *L.peruvianum* are a rich source of vitamin C. But, it is difficult to transfer these specific traits to cultivated tomato as they are governed by polygenes and existence of specific barriers in inter-specific hybridization with wild relatives. In this context, development of regeneration and transgenic protocols are highly essential. Tomato is very amenable to tissue culture and highly responsive to *in vitro* cultures. Standardization of in-*vitro* propagation protocols in this crop is also essential for the development of efficient transformation procedures. Until now different explants such as leaf discs (Mc Cormick *et al*, 1986), cotyledon and hypocotyls (Park *et al*, 2003) were used for in vitro regeneration and transformation. However, regenerative response is greatly dependent on the genotype. Though several protocols were developed for different varieties, work on regeneration protocol for PKM-1 is so far has not been accomplished. PKM-1 is an adaptable high yielding cultivar widely grown in A.P for its high acidity and is ideally suitable for long distance transport and is also mostly used as a parent for the development of green shoulder hybrids. Hence, development and standardization of in-*vitro* propagation protocols in this variety will lead to success in the production of transgenic tomato green shoulder hybrids resistant to biotic and abiotic stresses. Hence, keeping these points in view the standardization of in-*vitro* propagation protocols using hypocotyls and cotyledonary leaf explants in tomato cv.PKM-1 has been undertaken.

MATERIAL AND METHODS

The present study was carried out at Transgenic Laboratory of Dept. of Plant Physiology,

Table 1. Shoot initiation, multiplication and elongation in cotyledonary explants of tomato cv. PKM-1.

S.No.	Concentrations	Days taken for shoot bud initiation	No. of explants producing shoot Buds	Shooting frequency (%)	Mean no. of shoots / explant	Length of shoots (cm)
1	MS + BAP 1.0	15.6	3.3	(43.63) 47.61	1.6	2.32
2	MS + BAP 1.5	16.0	4.3	(51.88) 61.90	1.3	2.31
3	MS + BAP 2.0	15.3	3.6	(46.35) 52.37	1.0	2.10
4	MS + BAP 2.5	18.0	2.3	(35.26) 33.33	1.3	2.30
5	MS + Kinetin 0.5	14.6	3.3	(43.63) 47.61	1.6	2.74
6	MS + Kinetin 1.0	16.0	3.6	(46.35) 52.37	2.3	2.86
7	MS + Kinetin 1.5	15.3	2.3	(35.26) 33.33	1.3	2.51
8	MS + Kinetin 2.0	16.3	2.0	(32.31) 28.57	0.6	2.72
9	MS + Zeatin 0.5	15.0	3.3	(43.63) 47.61	0.6	2.50
10	MS + Zeatin 1.0	15.3	2.6	(38.10) 38.09	1.3	2.43
11	MS + Zeatin 1.5	15.6	2.3	(35.26) 33.33	1.6	2.50
12	MS + Zeatin 2.0	16.0	1.6	(29.19) 23.80	0.6	2.36
13	MS + BAP 1.0 + Kinetin 0.5	13.3	4.3	(51.88) 61.90	3.3	2.36
14	MS + BAP 1.5 + Kinetin 1.0	12.3	5.3	(60.78) 76.18	4.3	2.70
15	MS + BAP 2.0 + Kinetin 1.5	14.3	3.6	(46.35) 52.37	2.6	2.40
16	MS + BAP 2.5 + Kinetin 2.0	15.0	3.3	(43.63) 47.61	1.6	2.30
17	MS + BAP 0.25 + IBA 0.1	14.6	3.3	(43.63) 47.61	2.0	2.26
18	MS + BAP 0.5 + IBA 0.1	15.6	2.6	(38.10) 38.09	1.6	3.43
19	MS + BAP 1.0 + IBA 0.1	16.0	2.0	(32.31) 28.56	1.0	3.40
20	MS + BAP 2.0 + IBA 0.1	16.0	1.6	(29.19) 23.80	1.0	3.50
21	MS + BAP 0.5 + IAA 0.1	12.6	3.3	(43.63) 47.61	0.6	3.15
22	MS + BAP 1.0 + IAA 0.1	13.6	3.6	(46.35) 52.37	1.0	3.03
23	MS + BAP 1.5 + IAA 0.5	14.6	3.0	(40.87) 42.83	1.3	3.33
24	MS + BAP 2.0 + IAA 0.5	14.0	2.0	(32.29) 28.54	2.0	3.33
25	MS + Zeatin 0.5 + IAA 0.1	13.6	3.6	(46.35) 52.37	1.0	2.80
26	MS + Zeatin 1.0 + IAA 0.1	14.6	4.3	(51.88) 61.90	1.6	2.96
27	MS + Zeatin 1.5 + IAA 0.5	14.0	3.3	(40.64) 47.61	1.0	2.83
28	MS + Zeatin 2.0 + IAA 0.5	15.3	3.0	(40.87) 42.83	1.0	3.00
	(±) S.Em	0.39	0.37	3.22	0.34	0.05
	C.D at 5%	1.12	1.06	13.4	0.97	0.16

Note: Figures in parentheses represent arc sine transformed values. Observations were taken from seven explants.

RARS, Tirupati and Dept of Genetics and Plant breeding S.V. Agricultural College, Tirupati. The seeds of PKM-1 procured from Dept. of Horticulture, TNAU, Coimbatore were used for further investigation. The seeds were immersed in sterile double distilled water for 15 minutes and treated with Bavistin 1% solution for 20 minutes followed by thorough rinsing with sterilized water. One drop of Tween-20 was added to the seeds and shaked thoroughly for 5 min and thoroughly rinsed with sterile distilled water for 4-5 times. The seeds were taken in to laminar air flow cabinet, and treated with different concentrations of various surface sterilants for different intervals of time with occasional swirling. They were washed with 4-5 changes of sterile distilled water and were treated with 70% ethyl alcohol for 30 sec followed by washing for 4-5 times with double distilled water.

Table 2. Effect of different hormonal treatments on rooting in tomato cv. PKM-1.

S.No.	Concentration	Days taken for root initiation	No. of shoots producing roots	Frequency of rooting (%)	Mean no. of roots / shoot	Mean length of roots (cm)
1	MS + IBA 0.1	0	0	0	0	0
2	MS + IBA 0.2	15.0	0.6	(21.38) 13.3	3.3	2.2
3	MS + IBA 0.3	14.3	1.3	(31.04) 26.6	3.8	2.26
4	MS + IBA 0.4	15.6	1.0	(26.56) 20.0	2.6	2.43
5	MS + Kinetin 0.5	0	0	0	0	0
6	MS + Kinetin 1.0	19	3.58	(57.79) 71.6	7.0	6.33
7	MS + Kinetin 1.5	20.6	3.25	(53.72) 65.0	7.0	5.26
8	MS + Kinetin 2.0	21.3	3.08	(51.70) 61.6	6.3	4.96
9	MS + BAP 0.25 + IBA 0.1	15.3	3.53	(57.17) 70.6	8.6	3.83
10	MS + BAP 0.5+ IBA 0.1	16.0	3.16	(52.71) 63.3	7.9	3.50
11	MS + BAP 1.0 + IBA 0.5	16.0	2.64	(46.89) 53.3	7.4	3.23
12	MS + BAP 1.5 + IBA 0.5	15.6	2.52	(45.11) 50.2	6.9	2.93
13	MS + Kinetin 0.5+ IBA 0.1	20.3	1.6	(35.24) 33.3	4.3	2.80
14	MS + Kinetin 1.0+ IBA 0.1	21.3	1.3	(31.04) 26.6	5.3	2.76
15	MS + Kinetin 1.5+ IBA 0.5	22.3	0.6	(21.38) 13.3	4.0	2.93
16	MS + Kinetin 2.0+ IBA 0.5	21.0	0.3	(14.88) 6.6	3.3	2.80
	(±) S.Em	0.35	0.32	4.49	0.34	0.10
	C.D. at 5 %	1.01		12.96	0.98	0.29

Note: Figures in parentheses represent arc sine transformed values.
Observations were taken from five shootlets.

After sterilization the seeds were germinated on ½ M.S medium. Cotyledonary explants obtained from 10 days old seedlings were cultured on MS basal medium supplemented with BAP, Kinetin, Zeatin, IAA and IBA. The twenty eight combinations viz., MS + BAP 1.0, MS + BAP 1.5, MS + BAP 2.0, MS + BAP 2.5, MS + Kinetin 0.5, MS + Kinetin 1.0, MS + Kinetin 1.5, MS + Kinetin 2.0, MS + Zeatin 0.5, MS + Zeatin 1.0, MS + Zeatin 1.5, MS + Zeatin 2.0, MS + BAP 1.0 + Kinetin 0.5, MS + BAP 1.5 + Kinetin 1.0, MS + BAP 2.0 + Kinetin 1.5, MS + BAP 2.5 + Kinetin 2.0, MS + BAP 0.25 + IBA 0.1, MS + BAP 0.5 + IBA 0.1, MS + BAP 1.0 + IBA 0.1, MS + BAP 2.0 + IBA 0.1, MS + BAP 0.5 + IAA 0.1, MS + BAP 1.0 + IAA 0.1, MS + BAP 1.5 + IAA 0.5, MS + BAP 2.0 + IAA 0.5, MS + Zeatin 0.5 + IAA 0.1, MS + Zeatin 1.0 + IAA 0.1, MS + Zeatin 1.5 + IAA 0.5, MS + Zeatin 2.0 + IAA 0.5 were tried on the explants obtained from 10 days of *in vitro* seedlings for identifying better explant regeneration.

The same combinations were also tried for shoot initiation, multiplication and elongation for cotyledonary explants. The inoculated cultures were incubated in culture rack provided with white fluorescent tubes with a light intensity of 30-40 m moles under a 16 hour light and 8 hr dark photoperiod regime in a culture room whose temperature was maintained at 25 ± 2°C. The observations viz., time taken for shoot bud initiation, no. of explants producing shoot buds, shooting frequency (%), mean length of shoots and no. of days taken for shooting were recorded. The multiple shoot buds that initiated from the small areas on the cut surfaces of cotyledons were excised and sub cultured on to fresh medium periodically until they grew to a length of 3-4 cm. Then, the elongated shoots were transferred to sixteen different combinations of rooting medium viz., MS + IBA 0.1, MS + IBA 0.2, MS + IBA 0.3, MS + IBA 0.4, MS + Kinetin 0.5, MS + Kinetin 1.0, MS + Kinetin 1.5, MS + Kinetin 2.0, MS + BAP 0.25 + IBA 0.1,

Proliferation of multiple shoots from cotyledonary explants up on sub culturing when MS medium is supplemented with

BAP 1.5 + Kinetin 1.0 mg/L

BAP 1.0 + Kinetin 0.5 mg/L

BAP 2.0 + Kinetin 1.5 mg/L

Plate – 1

MS + BAP 0.5+ IBA 0.1, MS + BAP 1.0 + IBA 0.5, MS + BAP 1.5 + IBA 0.5, MS + Kinetin 0.5+ IBA 0.1, MS + Kinetin 1.0+ IBA 0.1, MS + Kinetin 1.5+ IBA 0.5, MS + Kinetin 2.0+ IBA 0.5. The data were also recorded on various rooting parameters viz., no. of shoots producing roots, rooting frequency (%), mean no. of roots / shoot, mean length of roots and no. of days taken for rooting. The *in vitro* rooted plantlets were removed from the culture vessels and the agar on the roots was gently washed off under tap water without damaging the roots. These plantlets were then transplanted to polythene bags containing autoclaved soil, sand, soilrite, and soil, sand and soilrite in the ratio of 1:2:1 and watered to field capacity. The bags were stapled with pin at the top to maintain high humidity and kept under white fluorescent lights at room temperature. After two weeks, the bags were opened and kept under the same conditions for another week. Then the plants were transferred to pots in the glass house. The statistical design for studying the better explants response and shoots regeneration among 28 treatments and for root regeneration among 16 treatments a completely randomized design was used. For each treatment, 10 bottles/plates/test-tubes constituting 4 replications were made. The data were analyzed for standard analysis of variance for various comparisons of the treatment differences.

RESULTS AND DISCUSSION

The data recorded on days taken for shoot bud initiation, no. of explants producing shoot buds, shooting frequency, mean no. of shoots / explant and length of the shoots when the cotyledonary leaf explants of tomato cv.PKM1 were cultured using MS basal medium with different hormonal concentrations of BAP, Kinetin, Zeatin or in combination with IAA and IBA were evaluated for their effects and presented in Table 1. The perusal of the data clearly showed significant differences between the treatments. Among different combinations, MS medium + BAP 1.5 mg/L+ Kinetin 1.0 mg/L recorded less no. days taken for shoot bud initiation (12.3 days) followed by MS medium + BAP 0.5 mg/L + IAA 0.1 mg/L (12.6 days), MS

Extent of Shoot elongation from cotyledonary explants when MS medium is supplemented with

BAP 2.0 + IBA 0.1 mg/L

BAP 0.5 + IBA 0.1 mg/L

BAP 1.0 + IBA 0.1 mg/L

Plate – 2

Table 3. Effect of different soil mineral mixtures on acclimatization and survival of regenerated plantlets in tomato cv. PKM-1.

S.NO	Soil mineral mixture	No. of days taken for acclimatization	Survival percentage
1	Soilrite	9.6	(68.02) 86
2	Sand	14.6	(30.0) 25
3	SoilSoil : Sand : Soilrite	13.3	(40.39) 42
4	(±) S.Em	12.3	(52.53) 63
	C.D at 5%	0.38	0.43
		1.3	1.51

Note: Figures in parentheses represent arc sine transformed values

Extent of root induction along with shoot regeneration in cotyledonary explants of PKM-1 when MS medium is supplemented with

BAP 0.25 mg/L + IBA 0.1 mg/L

BAP 0.5 mg/L + IBA 0.1 mg/L

BAP 1.0 mg/L + IBA 0.5 mg/L

Plate – 3

medium + BAP 1.0 mg/L + Kinetin 0.5 mg/L (13.3 days) compared to all other hormonal combinations. However, there was no significant difference between these treatments. Similar results were reported by Izadpanah and Khoshkui (1989) in tomato. The results of the present study further lead support to the fact that shoot regeneration and its influence by hormonal concentrations are genotype dependent and they vary with the type of genotype selected for the study.

For the parameter no. of explants producing shoot buds, the combination of MS medium + BAP 1.5 mg/L + Kinetin 1.0 mg/L recorded 5.3 out of 7 explants with shooting frequency of 76.18 % followed by MS medium + BAP 1.0 mg/L + Kinetin 0.5 mg/L and MS medium + Zeatin 1.0 mg/L + IAA 0.1 mg/L (4.3 explants/7 explants) with frequency of 61.90% compared to other treatments. However, there was no significant difference between these treatments. The hormonal concentration giving more mean no. of shoots/explant was MS medium + BAP 1.5 mg/L + Kinetin 1.0 mg/L (4.3 shoots) followed by MS medium + BAP 1.0 mg/L + Kinetin 0.5 mg/L (3.3 shoots); MS medium + BAP 2.0 mg/L + Kinetin 1.5 mg/L (2.6 shoots) (Plate 1). When the sub cultures were done in the same media, no. of shoots and the length of the shoots has been increased (Plate 2). Significant difference has been observed between these treatments.

Among different combinations used in the experiment, the highest shoot length was recorded when MS medium was fortified with BAP 2.0 mg/L + IBA 0.1 mg/L (3.5 cm) followed by BAP 0.5 mg/L + IBA 0.1 mg/L (3.43 cm) and BAP 1.0 mg/L + IBA 0.1 mg/L (3.40 cm) compared to other hormonal combination. However, the MS medium supplemented with BAP 1.5 mg/L + Kinetin 1.0 mg/L had taken lower no. of days for shoot bud initiation, high shooting frequency, higher mean no. of shoots/ explant with higher length of the shoot followed by BAP 1.0 + Kinetin 0.5 mg/L compared to all other hormonal combinations.

The *In-vitro* root induction studies for all the rooting parameters are presented in the Table 2 and showed significant differences

Extent of root length before acclimatization in cotyledonary Explants of PKM-1 when MS medium is supplemented with

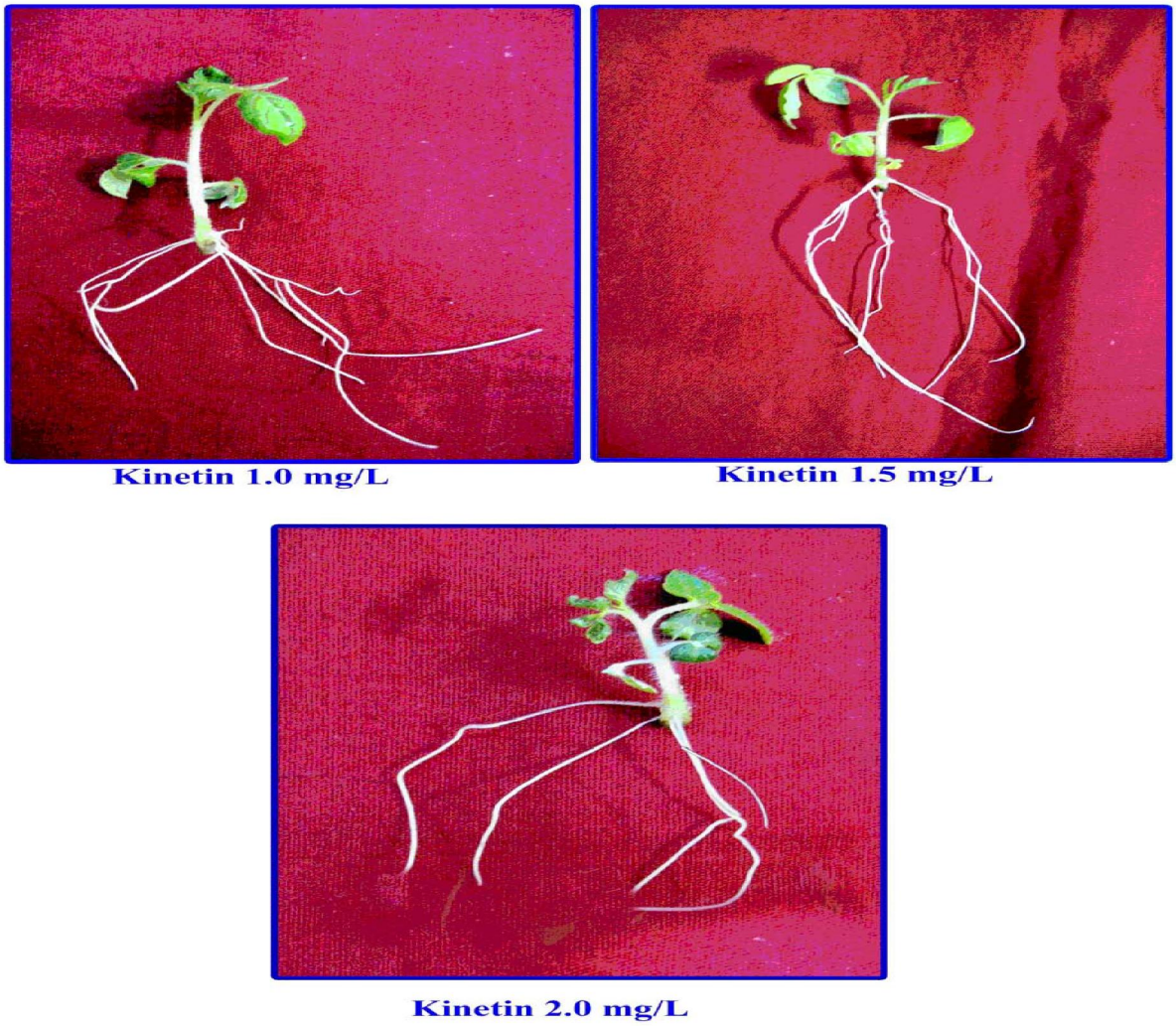

Plate – 4

among the treatments. Mean no. of days taken for root initiation was significantly lower when MS medium was supplemented with IBA 0.3 mg/L (14.3 days) followed by IBA 0.2 mg/L (15.0 days) and BAP 0.25 mg/L + IBA 0.1 mg/L (15.3 days) compared to all other combinations. MS medium with Kinetin 1.0 mg/L resulted in the highest no. of shoots (3.5 shoots/5 shoots) producing roots with the highest frequency of 71.6 % compared to all other treatments followed by BAP 0.25 mg/L + IBA 0.1 mg/L with rooting frequency 70.6 %, Kinetin 1.5 mg/L with rooting frequency 65 %. However, there was no significant difference between these treatments.

Among the various treatments, the combination i.e. MS medium + BAP 0.25 + IBA 0.1 mg/L recorded high mean no. of roots (8.6) followed by MS medium + BAP 0.5 mg/L + IBA 0.1 mg/L (7.9), MS medium + BAP 1.0 mg/L + IBA 0.5 mg/L (7.4) compared to other treatments (Plate 3). Similarly, MS medium fortified with 1.0 mg/L Kinetin recorded higher mean length of root of 6.33 cm followed by Kinetin 1.5 mg/L (5.26 cm) and Kinetin 2.0 mg/L (4.96 cm) (Plate 4). It is pertinent to note that significant differences were also observed between these treatments.

By and large, in the present study for root induction, MS medium + Kinetin 1.0 mg/L proved to be more successful combination recording more no. of shoots producing roots (3.3 Shoots/5 Shoots), maximum rooting frequency (71.6 %) with mean no. of roots/shoot (7.0) and high mean length of

Acclimatization of in vitro grown plants in polythene bags containing soilrite

Plate – 5

Estimation of rooted shoots of PKM-1 for ex vitro transfer

Plate - 6

roots (6.3 cm) although it has taken 19 days for root initiation which could be exploited for rapid multiplication of tomato cv.PKM1. Similar kind of results on root induction was reported by Polevaya *et al*. (1988) in tomato. Different workers have used different auxins for root induction with full strength MS medium (Mandal, 1999 and Soniya *et al*., 2001). Mostly in all rhizogenesis studies, auxins are mostly used due to their regeneration response. However, in the present study, both auxins (IBA) and cytokinins (Kinetin) were used alone or in combinations.

In addition to the shoot and root induction studies, the studies on acclimatization with the different soil mineral mixtures evaluated were presented in Table 3. Among all the treatments *in vitro* rooted plants established with 86 % success in poly bags containing soilrite mixture which took 9.6 days for acclimatization (Plate 5 and Plate 6) followed by soil : sand : soilrite (1:2:1) with 63 % and 12.3 days for acclimatization. Significant differences were also observed between these treatments. In contrast, Rao *et al.* (2007) reported 75-80% survival when plants were acclimatized with sand and soil mix (1:1) while Dwivedi *et al.* (1990) revealed that *in vitro* raised tomato plants grew normally when potted in soil.

In conclusion, the cotyledon regeneration system was proved to be an excellent method, as it has produced large number of regenerated tomato plants (86%) over a relatively shorter period i.e. (3 months). In cotyledons, shoot formation was rapid and prolific and a large proportion of these shoots were developed in to phenotypically normal fertile plants. This protocol is a genotype dependent one and it has provided a way for transformation of plants with desired gene of interest.

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