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Research Article

Pflp gene transformation in pomegranate (*Punica granatum* L.) resistance to bacterial blight disease (*Xanthomonas axonopodis* pv. *punicae*)

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Abstract
Pomegranate (*Punica granatum* L.) is a woody perennial fruit crop grown in arid zones of India, the state of Maharashtra being considered as pomegranate basket contributes to 70 % of the total area followed by Karnataka and Andhra Pradesh. Protection of crops against bacterial disease is an important issue in agricultural production. Pomegranate production in India is severely hampered by the high incidence of bacterial blight disease caused by *Xanthomonas axonopodis* pv. *punicae* is air borne, the conventional ways and means of controlling this disease have failed. Evolving a resistant genotype using resistant variety through conventional breeding may be a way out but it is a time consuming process. Transgenic approach appears to be promising to minimize the losses caused by disease. In the present investigation efficient protocols were developed to get healthy and well-formed plants from juvenile and mature-origin explants of the pomegranate cv. 'Bhagwa' and transformants with PFLP gene. One of the strategies to lead plants become resistant against bacterial pathogens is employing a transgene, like plant ferredoxin-like protein (PFLP). Different treatment combinations of hormonal concentrations were taken for leaf, petal, nodes and cotyledonary explants to standardize an efficient *in vitro* regeneration protocol and find out the best treatment for faster regeneration. *Agrobacterium tumefaciens* carrying gene pCAMBIA construct with the constitutive CaMV35S promoter, PFLP gene, terminator and *nptII* selectable marker (Kanamycin resistance), was used for transformation of explants. Putative transformants were identified on selection medium containing kanamycin at different concentration. Integration of transgene and expression at various levels were confirmed using PCR. Out of 4 putative transformants analyzed, three plants showed amplification for PFLP gene specific primer.

Key words
In vitro, *Pflp* gene, transformation, bacterial blight disease, putative transformants, *Agrobacterium tumefaciens*

Introduction

Pomegranate (*Punica granatum* L.) is a species of fruit-bearing deciduous shrub belonging to family *Punicaceae*. It is regarded as "vital cash crop" of an Indian farmer (Jadhav and Sharma, 2009). Among the different states growing pomegranate, Maharashtra is the largest producer occupying 2/3rd of total area in the country followed by Karnataka, Andhra Pradesh, Gujarat and Rajasthan. The fruit has a wide consumer preference for its attractive, juicy, sweet, acidic and refreshing arils. Pomegranate is good source of carbohydrates and minerals such as calcium, ironand sulphur. It is rich in vitamin C and citric acid is the predominant organic acid in pomegranate (Malhotra *et al.*, 1983).

Pomegranate is affected by many diseases. The bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae* has become an increasingly serious threat for pomegranate growers in the states of Andhra Pradesh, Maharastra and Karnataka. The disease was most severe in Karnataka and recorded to an extent of 60-90 % of incidence. The disease causes spots on leaves leading to defoliation, fruit spots and cankerous lesions on stem leading to death of plants in severe cases.

Genetic transformation studies for plants started during the early 1980's. This technology is a powerful tool to save the enormous losses caused by biotic and abiotic stresses in almost all the major crops. Transgenic crop plants have now emerged as an integral component of integrated crop management strategy and provide the much-needed strength and stability to the whole programme as well as deliver significant economic benefits (Bansal *et al*., 2004). Since genetic transformation has been achieved with considerable success, possibilities for the transfer of useful genes are becoming a reality (Ganapathi *et al*., 2001). Strategies to enhance plant disease resistance through genetic transformation have included expression of plant defense response pathway components (Keen, 1990) and genes encoding elicitors of defense response (Jaynes *et al*., 1987).

Plant Ferredoxin Like Protein (PFLP) is a ferredoxin-I containing a Ser and Thr-rich N-terminal signal peptide of 47 amino acids targeting to chloroplast and putative 2Fe-2S domain. This protein is involved in many redox reactions leading to the production of Reactive oxygen species (ROS). Ferredoxins are electron carriers in photosynthetic tissues and it is found that PFLP enhances ROS production, so as a result intensifies the harpin-mediated hypersensitive response (HR) (Dayakar *et al.*, 2003).

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Genetic engineering of any valuable crop requires an efficient regeneration protocol and transformation system. Due to the simplicity of the transformation system and precise integration of transgene, *Agrobacterium* Ti plasmid-based vectors continue to offer the best system for plant transformation (Veluthambi *et al*., 2003) and resultant plants are generally fertile (Aldemita and Hodges, 1996). Some Protocols for stable transformation of important plants through *Agrobacterium* with both disarmed and wild strains have been published during the last decades.

Materials and methods

The lab experiment was conducted in Tissue Culture Laboratory, Biotechnology Division, Indian Institute of Horticulture Research, Bangalore during 2012-13. The following steps were:

Explants collection: Leaves, cotyledons, petals and nodes of Pomegranate (*Punica granatum* cv.*Bhagwa*) were collected from the high yielding trees growing in the fields of division of fruit crop, Indian Institute of Horticulture Research, Bangalore.

Explants sterilization and inoculation: The explants collected were washed thoroughly in running tap water. Explants were treated with Tween-20 detergent for 10 minutes and washed thoroughly in sterilized water. Nodal twigs were trimmed and treated with 0.1% Cap50 for 10 minutes followed by distilled water wash. Explants were further sterilized with 0.1% $HgCl_2$ for 1-3 minutes. Leaves were trimmed and single nodes were separated and blotted (Table 1). The explants were inoculated on MS media supplemented with BAP and NAA ranging from 0 – 6 mg l^{-1}. Callus obtained from all the explants were subcultured on the same medium for callus proliferation.

The inoculated cultures were kept in dark condition for first 3 days and transferred to light condition in culture room. Cultures were maintained in culture room at a temperature of 25 to 280C, with a relative humidity of 60% under a photoperiodic regime of 16:8 light and dark hours. The illumination was provided by cool white fluorescent tubes at an intensity of 2000 Lux. Sub culturing First subculture of nodes was done after 7 days of inoculation and subsequent subculturing was done at an interval of 8-10 days. After 3rd subculturing nodes were shifted to 1/2 MS media for elongation. For petals and cotyledons first subculturing was done at 8 days of inoculation. The subsequent subculturing was done at 15-20 days of interval. For leaves first subculturing was done at 7 days after inoculation and the subsequent subculturing was done at 15-20 days of interval.

Agrobacterium-mediated Transformation: Leaf, node, petal and cotyledon calli were co-cultivated with *Agrobacterium tumefaciens strain* LBA4404 harbouring pCAMBIA 2301 binary vector containing *pflp* gene with *nptII* as selectable marker. The gene was driven by CaMV35S promoter for constitutive expression (Figure 1).

Plasmid pCAMBIA: In this research work for plant transformation, pCAMBIA containing the plant ferredoxin like protein gene was available at Indian Institute of Horticultural Research was used.

Maintenance and growth of Agrobacterium cultures: Agrobacterium tumefaciens was cultured in YEB medium containing 100mg ml^{-1} kanamycin at 28^0C for 24 hrs.

Agrobacterium Co-cultivation: The 30 to 60 day old calli were co-cultivated with different concentration of *Agrobacterium* culture (1 and 2 O.D) for different durations, from 4-8 minutes. Co cultivated callus were blotted on sterile blotting paper and placed on the callus induction medium. Co-cultivated cultures were kept in a dark condition for 3 days. After three days the calli were sub-cultured to media containing 25 mg ml^{-1} cefotaxime. It was kept for 7 days. This was placed under 16/8hrs photoperiod at 25^{o}C in growth chamber.

Selection of transformants: After three days of Co-cultivation the shoot tips were transferred to selection medium containing cefotaxime (250mg ml^{-1}) and kanamycin (100mg m l^{-1}). Cultures were periodically subcultured to obtain healthy putative transformed plants.

Confirmation of integration of the transgene: Genomic DNA of control and putatively transformed plants were isolated from pomegranate plants, to confirm the integration of PFLP gene using PCR.

Isolation of plant genomic DNA by CTAB method: Cetyl trimethyl ammonium bromide is a detergent which is used along with other reagents to liberate nucleic acids from the cell. This is an efficient method for isolating plant genomic DNA from leaf tissues. The high molecular weight DNA obtained is purified by phenol: chloroform method to remove proteins and other plant debris.

Polymerase chain reaction is a very simple method for *in vitro* amplification of specific nucleic acids using Taq DNA polymerase and short stretch of oligonucleotides (primers), which are specific to the DNA to be amplified. The confirmation of the presence of the transgene can be done by PCR amplification of the PFLP gene by using gene specific primers and visualizing the amplified DNA on electrophoretic gel.

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Results and discussion

Bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae*, once a disease of minor importance has became major constraint since 2002. All conventional ways of controlling this disease have failed. Transgenic approach appears to be a promising to minimize the loss caused by disease. *Agrobacterium* mediated transformation is the most preferred method of plant transformation (Wordragen and Dons, 1992). However, a severe impediment to the applicability of *Agrobacterium* mediated transformation approach with many plant species is lack of efficient and reliable transformation and regeneration system.

The explants were maintained in polyhouse to minimize the contamination *in vitro*. Chemicals like Tween20, Cap50, $HgCl_2$ and Sodium hypochlorite were used for sterilization. There was significant difference was observed for percentage of browning and also for percentage of contamination free plants. Petal explant sterilized with HgCl2 (0.10 %) for 1min showed highest percentage of contaimination free plants which is on par with cotyledonary explant. Maximum percentage of browning was observed in leaf explant and minimum percentage of browning was observed in cotyledonary explants (Table 2).

In the present study callus initiation started 30 – 45 DAI in leaf and cotyledonary explants whereas in petal explants it took 4 – 6 months for callus regeneration. The addition of 1.8mg l^{-1}BAP and 0.4 mg l^{-1} NAA to the MS media stimulated 90 % callus regeneration and shoot regeneration in nodes, while 2mg l^{-1}BAP and 2 mg l^{-1} NAA produced 95 % callus regeneration and shoot regeneration in leaves. Deepika and Kanwar (2010) reported the best medium for leaf callus induction was MS medium supplemented with 8.0 µM NAA and 9µM kinetin. MS medium supplemented with 2mg l^{-1}BAP and 4 mg l^{-1} NAA was found to be the best combination for callus regeneration and shoot regeneration in petal where as in cotyledons MS medium supplemented with 2.5 mg l^{-1}BAP and 1.5 mg l^{-1} NAA (Table 3).

Hence, in the present study, a systematic investigation was carried on the effect of some major factors involved in the development of an efficient *in vitro* regeneration and transformation procedure for pomegranate cv. Bhagwa. A reliable and efficient protocol for *in vitro* regeneration and transformation of pomegranate cv. Bhagwa was developed. PFLP gene was transferred into pomegranate using the above protocol. Putative transformants were obtained on selection medium. Transgene insertion and expression were confirmed using molecular techniques.

Agrobacterium mediated transformation: Investigating the influence of different concentration of *Agrobacterium* cultures and duration of inoculation OD_{600} of 0.2 gave optimum results with co-cultivation period of 8 min (Table 4).

In fruit trees, transformation rate is very low, besides cultivar and developmental stage of explant are other factors influencing transformation rate (Petri and Burgos, 2005). *Agrobacterium* mediated transformation offers remarkable advantages over direct gene transfer methodologies. It reduces the copy number of the transgene, potentially leading to fewer problems with transgene co-suppression and instability.

A. tumefaciens strain LBA4404 has been used for many plant transformations because the elimination of LBA4404 from plant tissues is relatively easy at low concentration of antibiotics (Maheswaran *et al*., 1992). So far only one report has been known on *A. tumefaciens* mediated transformation of dwarf pomegranate (Terakami *et al*., 2007). In the present study we were able to establish an *A. tumefaciens*-mediated transformation method of pomegranate using *Agrobacterium* strain LBA4404.

Fully callused petal, leaves, and cotyledons were inoculated with overnight grown *A. tumefaciens* LBA4404 culture for 8min because of more chances to enter the gene in callus and then incubated for 3 days in dark, then they were transferred to cefotaxime for 3 days then shifted to the media containing 100 mg l^{-1} kanamycin and 250 mg l^{-1} of cefotaxime for selection of transformants.

Selection of transformants: The co-cultivated leaf, cotyledon and petal calli were cultured on cefotoxime media (250 mg ml^{-1}) for 3 days, then they were sub cultured on the media containing cefotaxime and kanamycin with concentration of 250 mg ml^{-1} and 100 mg ml^{-1} respectively (Plate 1).

The control plants obtained from leaf, cotyledon and petal calli were died and only putative transformants were regenerated from the media after 45-60 days of sub culturing.

The co-cultivated explants were regenerated on kanamycin selective medium. After 5-6 weeks putative transgenic shoots were obtained from the transformed explants. In the control untransformed explants, leaves were withered on the selection media and died due to susceptibility to kanamycin selective medium.

Confirmation of PFLP gene in putative Transformants: The genomic DNA was isolated by CTAB method and confirmation was done by PCR analysis.

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Out of 4 putative transformants, two cotyledon derived plants and one leaf derived plant shown presence of PFLP gene at 460bp and one leaf derived plant shown absence of PFLP gene.

The presence of the transgene PFLP into the plant genome was confirmed by PCR analysis. PCR was performed using gene specific PFLP primers and 460bp corresponding to the amplified internal fragment of PFLP gene were detected (Plate 2). The amplified products were observed in all the transformed plants tested, confirming the presence of transgene. No amplified product was observed in case of the untransformed plants (control).

Conclusion

The presence of the transgene PFLP into the plant genome was confirmed by PCR analysis. PCR was performed using gene specific PFLP primers and 460bp corresponding to the amplified internal fragment of PFLP gene were detected. Further, putative transformants need to be confirmed for the integration of *pflp* gene through RT-PCR and Southern analysis, expression analysis by ELISA and Western blotting. Finally to challenge the pathogen in the green house condition screening has to be done to know the resistance of transformed plants to the bacterial blight disease caused by *Xanthomonas axonopodis* pv. *punicae*.

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Table 1. Different sterilization treatments used for explants of pomegranate

Sl. No.	Cap 50 (0.1%) mins	Tween-20 (1%) mins	$HgCl_2$ (0.1%) mins
1	10	4	1
2	10	4	2
3	10	4	3
4	10	8	1
5	10	8	2
6	10	8	3
7	10	10	1
8	10	10	2
9	10	10	3
10	15	4	1
11	15	4	2
12	15	4	3
13	15	8	1
14	15	8	2
15	15	8	3
16	15	10	1
17	15	10	2
18	15	10	3

Table 2. Treatments used for sterilization of different explants of pomegranate

Sl. No.	Type of explant	Type of chemical used			Percentage of Contamination free plants	Percentage of browning
		Tween20	Cap50	$HgCl_2$		
		1%	0.10%	0.10%		
1	Nodes	10min	15min	1min	94(75.82)	8(16.43)
2	Leaves	10min	15min	1min	90(71.56)	30(33.21)
3	Petals	-	-	1min	98(81.87)	13(21.13)
4	Cotyledons	-	-	1min	96(78.46)	5(12.92)
SEM					0.868	0.575
CD (1%)					4.121	2.732
F-TEST					**	**

**= significant at P=0.01%
Note: Figure in the parenthesis indicates arc sin transformed value.

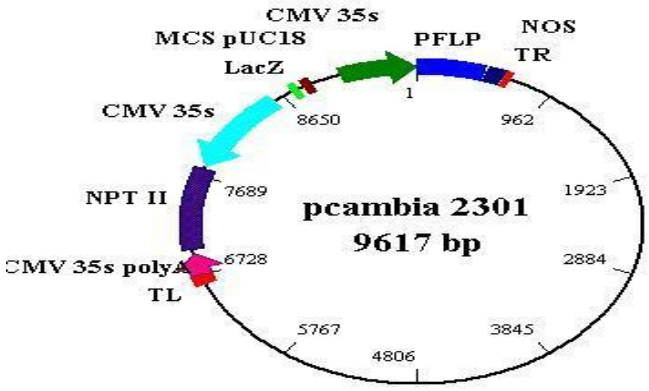

Fig. 1. The vector pCAMBIA 2301 containing the PFLP gene (constructed at IIHR)

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Table 3. Percentage of callus formation from different explants of pomegranate using different concentrations of growth hormones

S. No.	Explant	Media		% of Callus Formation
		BAP(mg l^{-1})	NAA(mg l^{-1})	
1	Node	1.8	0.4	90
2	Leaf	2	2	95
3	Petal	2	4	96
4	Cotyledon	2.5	1.5	96

Table 4. Standardization of Co-cultivation duration of Leaf, Cotyledon and Petal explants at different OD_{600}

Type of explants	OD_{600}	Co-cultivation period min.	No. of shoots regenerated	No. of shoots rooting
Leaf	0.1	4	-	-
	0.1	8	-	-
	0.2	4	-	-
	0.2	8	10	7
Cotyledons	0.1	4	-	-
	0.1	8	-	-
	0.2	4	-	-
	0.2	8	4	3
Petals	0.1	4	-	-
	0.1	8	-	-
	0.2	4	-	-
	0.2	8	1	-
S.EM			**0.22**	**0.21**
CD (1%)			**0.92**	**0.93**
F Test			**	**

**= significant at P=0.01%
(Note: Figure in the parenthesis indicates arc sin transformed value.)

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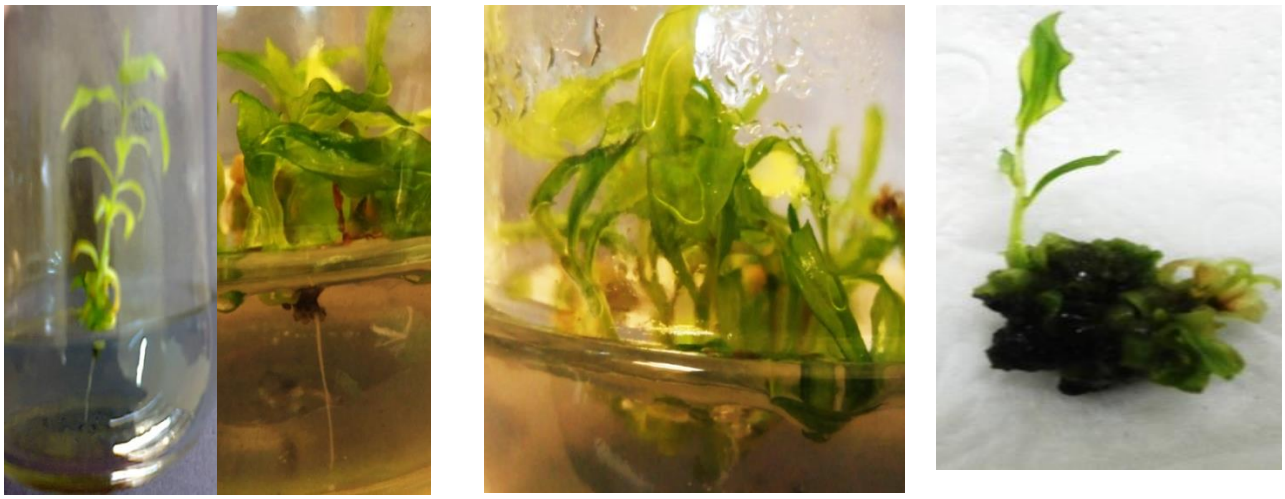

A. Rooting response of shoot regenerated from leaf calli

B. Multiple shoots regenerated from leaf calli

C. Shoot regeneration from Cotyledonary calli

Plate 1. Shoot regeneration and rooting response of putative transformants

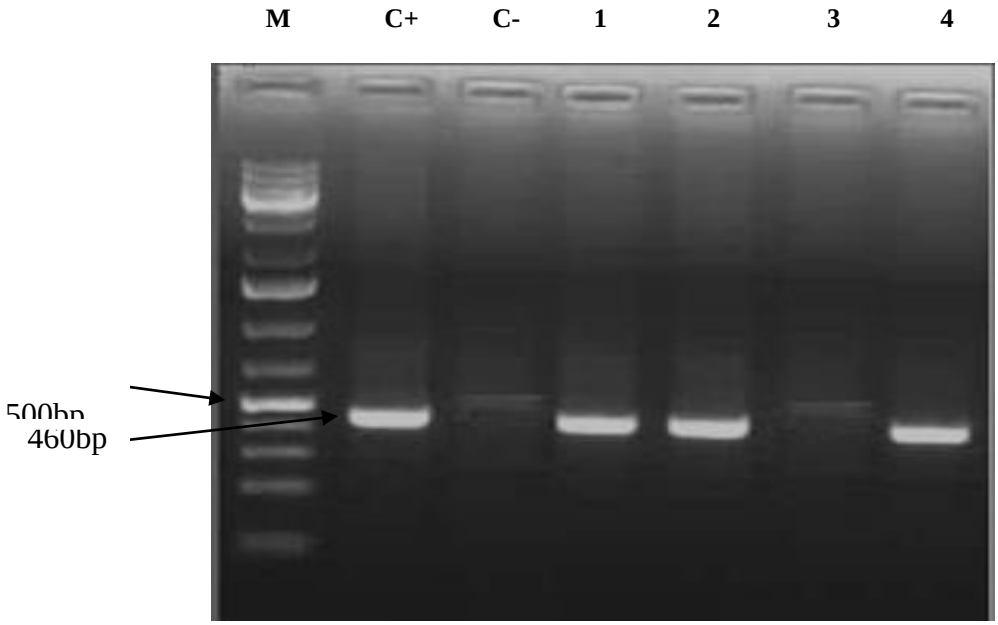

Plate 2. PCR screening of the putative transformants using PFLP specific primer

(M-100bp Marker, C+ Positive Control, C- Negative Control, Lane1 and 2 putative transformants from the cotyledon explant, Lane 3 and 4 putative transformants from the leaf explant)