***Pflp* Gene transformation in Pomegranate (*Punica Granatum* L.) resistance to Bacterial Blight disease (*Xanthomonas axonopodis* pv. *Punicae*)**

AKSHATA HOSAMANI1\*, SUKHADA MOHANDAS2, MANJULA V3, PRAKASH KERURE4. AND GEETHA, M.S5

*Division of Plant Biotechnology, Indian Institute of Horticultural Research, Hesaraghatta, Bengaluru-560089 (Karnataka)*

**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, 3 plants showed amplification for PFLP gene specific primer.

**Keywords:** *Invitro*, *Pflp* gene, Transformation, Bacterial Blight Disease, Putative transformants, *Agrobacterium tumefaciens*

*(1\* M.Sc. Work and Author, AAO, Dept. of Agriculture, GOK, 2 Principal Scientist and Guide, Indian Institute of Horticultural Research, Hessarghatta, Bengaluru, 4 Assistant Professor of Horticulture and correspondent, ICAR-Krishi Vigyan Kendra, Chitradurga, Karnataka)*

**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 and 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).

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 ofPomegranate (*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% HgCl2 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.

**Table 1: Different sterilization treatments used for explants of pomegranate**

|  |  |  |  |
| --- | --- | --- | --- |
| **Sl. No.** | **Cap 50 (0.1%) Mins** | **Tween-20 (1%) Mins** | **HgCl2 (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 |

***Agrobacterium*-mediated Transformation:**

**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.



**Figure 1: The vector pCAMBIA 2301 containing the PFLP gene (constructed at IIHR)**

**Maintenance and growth of *Agrobacterium* cultures:**

*Agrobacterium* *tumefaciens* was cultured in YEB medium containing 100mg ml-1 kanamycin at 280C 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 subcultured to media containing 25 mg ml-1 cefotaxime. It was kept for 7 days. This was placed under 16/8hrs photoperiod at 250C 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.

#### RESULTS AND DISCUSSIONS:

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, HgCl2 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).

**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 | HgCl2 |
| 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) |
| S.EM |  |  |  |  | 0.868 | 0.575 |
| CD (1%) |  |  |  |  | 4.121 | 2.732 |
| F-TEST |  |  |  |  | \*\* | \*\* |

NS= NON SIGNIFICANT, \* SIGNIFICANT AT P=0.05O%, \*\*= SIGNIFICANT AT P=0.01%

Note: Figure in the parenthesis indicate arc sin transformed value.

In the present study callus initiation started 30 – 45 DAI in leaf and cotyledonary explants whereas in petal explants it took 4 – 6months for callus regeneration. The addition of 1.8mg l-1BAP and 0.4 mg l-1 NAA to the MS media stimulated 90% callus regeneration and shoot regeneration in nodes, while 2mg l-1BAP 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-1BAP 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-1BAP and 1.5 mg l-1 NAA(Table 3).

**Table 3:** Percentage Of Callus Formation From Different Explants Of Pomegranate using Different Concentrations Of Growth Hormones.

|  |  |  |  |
| --- | --- | --- | --- |
| **Sl. 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 |

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 OD600 of 0.2 gave optimum results with co-cultivation period of 8min (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.

**Table 4: Standardization of Co-cultivation duration of Leaf, Cotyledon and Petal explants at different OD600**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Type of explants** | **OD600** | **Cocultivation 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** |
|  |  |  | \*\* | \*\* |

NS= NON SIGNIFICANT, \* SIGNIFICANT AT P=0.05O%, \*\*= SIGNIFICANT AT P=0.01%

(Note: Figure in the parenthesis indicates arc sin transformed value.)

**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.

|  |  |  |
| --- | --- | --- |
|  |  |  |
| 1. **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**

**Confirmation of PFLP gene in putative Transformants**

The genomic DNA was isolated from both control and regenerated plants by CTAB method and confirmation was done by PCR analysis.

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).

 **M C+ C- 1 2 3 4**



460bp bp

 500bp

**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)

**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 challenging 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*.

**REFERENCES:**

Aldemita, R. A. and Hodges, T. K., 1996, *Agrobacterium* *tumefaciens* mediated transformation of *japonica* and *indica* rice varieties. *Planta*, **199**: 612-612.

Bansal, K. C., Singh, N. K., Prasanna, B. P., Tiwari, S. P. and Dhillon, B. S., 2004, Transgenic crops: Status and prospects in Indian agriculture. In: National conference on ‘Transgenics in Indian agriculture’. 9 and 10thMarch: 17-30**.**

Dayakar, B. V., Lin, H. J., Chen, C. H., Ger, M. J., Lee, B. H., Pai, C. H., Chow, D., Huang, H. E., Hwang, S. Y., Chung, M. C. and Feng, T.Y., 2003, Ferredoxin from sweet pepper (*Capsicum annuum L*,) intensifying harpin Pss-mediated hypersensitive response shows an enhanced production of active oxygen species (AOS). *Plant Mol. Biol*, **51:** 913–924.

Deepika, R. and Kanwar, K., 2010, In vitro regeneration of Punica granatum L. plants from different juvenile explants. J. Fruit Ornam. *Plant Res*., **18**(1): 5-22.

Ganapathi, T. R., Higgs, N. S., Balint –Kurti, P. J., Arntzen, C.J., May, G, D. and Vaneck , J. M., 2001, *Agrobacterium* – mediated transformation of the embryogenic cell suspensions of the banana cultivars Rasthali (AAB). *Plant Cell Rep*., **20:**157-162.

Jadhav, V. T. and Sharma, K. K., 2009, Integrated management of diseases in pomegranate.

Jaynes, J. M., Xanthopoulos, K. G., Destefano-Beltran, L. and Dodds, J. H., 1987, Increasing bacterial disease resistance in plants utilizing antibacterial genes from insects. *Bioassays,* **6:** 263-270.

Keen, N. T., 1990, Gene-for-gene complementarity in plant-pathogen interactions. *Annu Rev Genet,* **24:** 447–463.

Maheswaran, G.M., Welander, M., Hutchinson, J.F., Graham, M.W., and Richards, D., 1992, Transformation of apple rootstock M26 with *Agrobacterium tumefaciens*. *J. Plant Physiol*., **139**:560–568.

Malhotra, N. K., Khajuria, H. N. and Jawanda., 1983, Studies on physico-chemical characters *Mediterranean Fruits,* Univ. Agric. Sci., Dharwad, June 23-27, pp. 48-52. of pomegranate cultivars II. Chemical characters. *Punjab Horti. J.*, **23 :** 158.Paper Presented In : *2nd Inter. Symp. Pomegranate and minor including*

PETRI, C and BURGOS, L., 2005, Transformation of fruit trees. Useful breeding tool or continued future prospect? *Transgenic Res*., **14**:15–26.

Terakami, S., Matsuta, N., Yamamoto, T., Sugaya, S., Gemma, H. and Soejima, J., 2007, *Agrobacterium*-mediated transformation of the dwarf pomegranate (*Punica granatum* L. var. Nana). *Plant Cell Rep,* **26:** 1243-1251.

Veluthambi, K., Gupta, K. A. and Sharma, A., 2003. The current status of plant transformation technologies. *Curr. Sci*, **84 (3)**: 368-379.

Wordragen, M. F. and Dons, H. J. M., 1992, *Agrobacterium*-mediated transformation of recalcitrant corps. *Plant Mol. Biol. Rep*, **10**:12-36.