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Research Article

TOWARDS ENGINEERING DROUGHT TOLERANCE IN TOMATO

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Abstract: Drought is one of the major abiotic stresses limiting tomato productivity and progress in development of drought tolerant tomato varieties is slow due to complex nature of tolerance mechanisms. Genetic engineering seems to be a viable approach for genetic manipulation of drought tolerance related traits in tomato. The present study was aimed at developing drought tolerant tomato (PKM 1) plants exhibiting enhanced expression of *EcNAC67* (a transcription factor controlling drought/salinity tolerance in finger millet) through genetic engineering. Seeds of PKM 1 tomato were germinated on MS medium and 7 - 9 days old cotyledonary leaves were used as explants for co-cultivation with *Agrobacterium* harboring a plant transformation vector pCAMBIA1300 engineered with *EcNAC67*. Co-cultivated explants were subjected to selection on media containing Hygromycin (10 mg/L) and putative transgenic plants were regenerated on MS+B5 media containing Zeatin at 1 mg/L concentration. Regenerated shoots were transferred to rooting media containing IBA (1 mg/L) and rooted plants were hardened and transferred to greenhouse for establishment. Putative transgenic (T0) tomato plants were screened through PCR analysis using *EcNAC67* specific primers which confirmed the presence of transgene.

Keywords: Tomato, Drought tolerance, NAC, *Agrobacterium*-mediated transformation

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Introduction

Tomato (*Solanum lycopersicum* L.) is extensively cultivated in tropical, sub-tropical and temperate areas globally with an area of 7.6 m ha with a total production of around 183.9 million tonnes [1]. In India, tomato is one of the most commonly consumed solanaceous vegetable next to Potato and Onion. Tomato is gaining remarkable acceptance among consumers during recent years due to possession of bioactive compounds such as lycopene exhibiting anti-oxidative activities and anti-cancer functions [2,3]. Among the various abiotic stresses affecting tomato productivity, drought, salinity and high temperature stresses stand at the top affecting both quality and production in India [4]. These abiotic factors affect several physiological and biochemical processes and thereby causing significant yield loss to the farmers [5]. Effect of these stresses will be more pronounced when these stresses coincide with the reproductive stage [6].

There exists limited genetic variation for drought/salinity tolerance related traits in the cultivated tomato germplasm and hence limited attempts have been made to understand genetic and molecular basis of drought/salinity tolerance traits in *Solanum lycopersicum* [7]. Thus, development of drought/salinity tolerant tomato through conventional breeding methods seems to be slow and necessitated adaption of novel strategies like genetic engineering. Genetic engineering is a promising approach for improving stress tolerance in crop plants by introduction of one or few genes from distantly related organisms [8], which will contribute in reducing the yield loss under stress conditions. First successful report on genetic transformation in tomato was reported during 1986 [9], and transformation efficiency in tomato is reported to be ranging from 7 % to 48 % [10-17]. Only limited attempts have been made towards genetic engineering of drought tolerance in tomato due to limited availability of major effect candidate genes. Mining of already reported information in the public repository seems to be a viable approach to select right candidate gene(s) for genetic engineering strategy. Our earlier experiments on salinity responsive transcriptome profiling in a set of contrasting finger millet genotypes *viz*., CO 12 (susceptible) and Trichy 1 (tolerant), through RNA-sequencing resulted in the identification of a novel NAC homolog namely, EcNAC 67 [18] and found to contribute for enhanced tolerance against drought/salinity in rice. Based on the above facts, the present study was formulated to develop transgenic tomato plants (PKM 1) engineered with *EcNAC67* through *Agrobacterium*-mediated transformation.

Materials and methods

Materials used

Genetically pure seeds of tomato (cv. PKM-1) were obtained from Department of Vegetable Crops, HC&RI, TNAU, Coimbatore and used. A plant transformation vector pCAMBIA1300 harboring the candidate gene namely *EcNAC67* from a saline tolerant finger millet genotype "Trichy 1" developed earlier in our laboratory [19] was used in this study. Single colonies of *Agrobacterium tumefaciens* strain LBA4404 harboring pCAMBIA1300-*EcNAC67* construct were identified by colony PCR using primers specific to CaMV35S, hyg; *EcNAC67* and Vir. Restriction digestion of the putative positive plasmids carrying the insert *EcNAC67* was carried out using the restriction enzymes *BamHI* and *KpnI*. The digested products were resolved in 1 % agarose gel and the size of the insert released was examined under UV.

Genetic transformation of PKM-1 using *Agrobacterium* harboring pCAMBIA1300-*EcNAC67*

Genetically pure seeds of cv. PKM-1 were surface sterilized using sterile water containing two drops of Tween 20 for 5 min with vigorous shaking, followed by 70 % ethanol treatment for 5 minutes. The seeds were then treated with 2 % Sodium hypochlorite for 5 min with occasional swirling followed by washes with sterile water, thrice. The seeds were blot dried on a sterile tissue paper and placed on half strength MS medium [20], and kept in dark for 3 days at 25 °C for germination and then transferred to 16/8 hr. light/dark condition maintained in the culture room.

Pre-culture

Cotyledonary explants were collected from 7-9-day old in-vitro grown seedlings. The distal and proximal ends (1-2 mm) were cut off and the explants were cut into two pieces before placing them on the pre-culture medium (MS medium modified with Gamborg vitamins and supplemented with 1.0 mg/L zeatin). Cotyledonary explants were placed in such a way that the abaxial side was in direct contact with medium two days prior to co-cultivation and maintained at 25 °C under 16 h light and 8 h dark condition.

Co-cultivation and Hygromycin sensitivity test

Tomato cotyledons were co-cultivated with *Agrobacterium* strain LBA4404 harbouring a binary vector pCAMBIA1300 engineered with *EcNAC67*. Further, hygromycin sensitivity test was carried out to find out the concentration of hygromycin required for inhibition of non-transformed plants. This was done by culturing the cotyledonary explants (without co-cultivation) on selection medium containing hygromycin at 7, 10 and 12 mg/L concentration and the lethal dosage causing yellowing and browning of leaf bits was identified.

Selection and regeneration

After co-cultivation, infected cotyledons were transferred on to shoot regeneration medium (MS-B5 medium containing 1 mg/L zeatin) supplemented with 250 mg/L of cefotaxime. They were sub-cultured onto a fresh medium at 15 days intervals, containing required quantity of hygromycin until they reach 1 cm long. Regenerated shoots were maintained under selection medium with antibiotics. They were maintained in same media composition until they reached 2-3 cm long before transferring into rooting medium.

Rooting, hardening and acclimatization of plantlets

Well-developed shoots were carefully transferred to medium containing half strength MS + 15 g sucrose + 1 mg/L of IBA + 0.8 % of agar for rooting and maintained under 16 hours light and 8 hours dark. Well rooted plants were hardened in greenhouse in small cups containing autoclaved coconut peat mixture covered with a polythene cover to maintain humidity. Well-established plants were transplanted into bigger pots and maintained under transgenic greenhouse conditions.

Molecular confirmation of the transformants

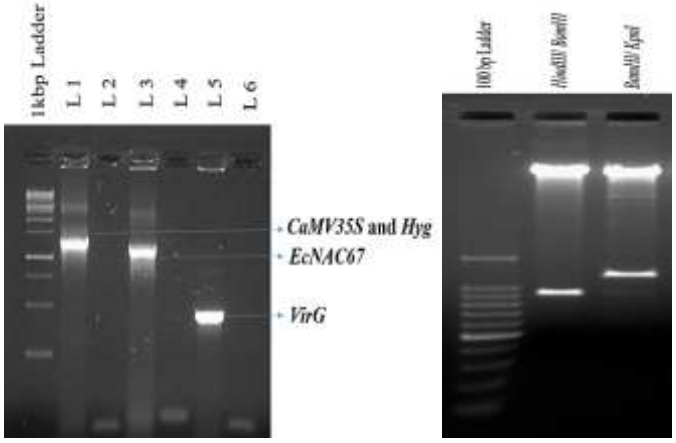
To confirm the presence of the *EcNAC67* in the putative transgenic plants, genomic DNA was isolated from the leaf tissues of both non-transgenic and transgenic plants following modified CTAB protocol [21] and used for PCR analysis. Plasmid DNA (pCAMBIA1300) was used as a positive control.

Results and discussion

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops worldwide. It is considered as a model crop for genetic and genomic studies due to its short generation time, smaller genome size and availability of standardized genetic transformation technologies [22]. Drought is one of the most significant abiotic stresses limiting growth, development and productivity of tomato to a greater extent up to 70% [23]. Hence developing an abiotic stress tolerant tomato is of high priority research worldwide. Although conventional breeding approaches like pedigree breeding, mutation breeding and heterosis breeding have enabled us to develop several high yielding tomato genotypes, progress in genetic enhancement of drought tolerance in tomato is limited due to narrow genetic variation for drought tolerance related traits in tomato germplasm and also due to complex and polygenic nature of drought tolerance mechanisms. During recent years, genetic engineering strategy has been successfully used in developing transgenic crop plants exhibiting enhanced tolerance against biotic/abiotic stresses, enhanced nutritional quality and other complex traits [24, 25]. Genetic engineering of transcription factors has been demonstrated to be a successful method of manipulating complex traits when compared to modification of individual genes regulating metabolic pathways because TFs modulate expression of hundreds of downstream genes involved in different metabolic pathways [26]. The NAC family of proteins (NAM-No apical meristem; ATAF - Arabidopsis transcription activation factor; CUC - Cup-shaped cotyledon) is one of the largest plant-specific transcription factors containing a highly conserved N-terminal DNA-binding domain, a nuclear localization signal sequence, and a variable C-terminal domain [27]. NAC family of genes were reported to be involved in modulating responses against various biotic and abiotic stresses which makes them potential for improving biotic and abiotic stress tolerance [28]. A NAC transcription factor JUNGBRUNNEN as reported to enhance drought tolerance in tomato [29]. In one of our earlier studies, transgenic rice plants harboring *EcNAC67* showed enhanced tolerance against drought and salinity under greenhouse conditions. Upon drought stress, transgenic lines were found to maintain higher relative water content and lesser reduction in grain yield when compared to non-transgenic ASD16 plants. Based on the above reports, it was planned to engineer *EcNAC67* into tomato (PKM-1) for enhancing its tolerance against drought/salinity.

Confirming the presence of *EcNAC67* in *Agrobacterium tumefaciens*

Agrobacterium strain LBA4404 harboring a binary vector pCAMBIA1300 engineered with *EcNAC67* were confirmed through colony PCR analysis using CaMV35S and hpt primers which produced an amplicon of 1300 bp fragment. Colony PCR analysis using primers specific to *EcNAC67* and vir genes resulted in the amplification of expected amplicon of 1178 bp and 440 bp fragments respectively which confirmed the presence of intact transgene cassette [Fig-1a]. Restriction digestion analysis of pCAMBIA1300 harboring intact *EcNAC67* using *HindIII*/*BamHI* resulted in the release of rd29A promoter (953 bp fragment) and double digestion with *BamHI*/*KpnI* released 1178 bp transgene *EcNAC67* fragment [Fig-1b]. These results indicate pCAMBIA1300 harboring intact fragment (1178 bp) of *EcNAC67*.



A. Colony PCR B. Restriction Digestion

Fig-1 Colony PCR and restriction digestion analysis of *A. tumefaciens* strain LBA4404 harboring pCAMBIA1300 carrying *EcNAC67*. A) Agarose gel electrophoresis of PCR amplified products using CaMV35S/hpt specific primers (Lane 1), *EcNAC67* specific primers (Lane 3) and VirG specific primers (Lane 5); B) Restriction digestion analysis of pCAMBIA1300 using *HindIII/BamHI* (Lane1) and *BamHI/Kpn1* (Lane 2)

Generation of transgenic tomato (PKM 1) plants engineered with *EcNAC67*

Agrobacterium mediated transformation method favors stable integration of single copy of the transgene when compared to direct transformation methods [30]. Compact lush green cotyledonary leaves collected from 7-9 days old seedlings were cultured on a pre-culture medium containing 1 mg/L zeatin for 2 days and co-cultivated with *Agrobacterium* strain LBA 4404 harboring pCAMBIA1300+*EcNAC67* [Fig-2a]. Optimized concentration of selection agent is one of the determinant factors in genetic transformation of plants [31]. Optimal concentration of hygromycin is required to for efficient selection of transformed cells [32]. We used hygromycin as a selectable marker to distinguish between the transformants and non-transformants as it was proven that hygromycin kills the non-transformed cells more quickly as compared to kanamycin [33, 34]. To evaluate the optimum concentration of hygromycin for tomato cv PKM-1, cotyledonary leaves of nine days old in vitro grown tomato were placed on MS

medium supplemented with three different levels of hygromycin viz., 7 mg/L,10 mg/L and 12 mg/L. Sensitivity test revealed that hygromycin @ 10 mg/L was found to be most effective which suggested the lower internal resistance of PKM 1to hygromycin as compared to other cultivars used by other researchers. Co-cultivated explants were washed with cefotaxime solution (250 mg/L) and placed on a selection media containing hygromycin (10 mg/L). Browning of non-transformed explants was noticed during the 1st selection itself. Putatively transformed explants remained green on selection media containing hygromycin (10 mg/L). Survived explants were further sub-cultured on selection media at every 15 days interval. Selection pressure was employed during every subculture and the non-transformed explants were eliminated. Regeneration of shoots was noticed 30 days after first selection upon addition of zeatin (1 mg/L). Putative transgenic plants were regenerated and rooted using appropriate hormonal combinations and sent for hardening under greenhouse condition [Fig-2b]. Putative T0 transgenic tomato plants were screened for the presence of transgene by PCR analysis using *EcNAC67* specific primers (NAC-F + NAC-R). Results of PCR analysis showed the presence of the transgene by producing an amplicon of 1178 bp [Fig-3]. Further molecular studies using southern hybridization and RT-PCR analysis will give information on copy number and expression of transgene(s). Developed transgenic plants will serve as a potential genetic material for unraveling molecular networks modulated by NAC67 transcription factor leading to enhanced drought/salinity tolerance.

A. Co-cultivated PKM-1 cotyledon on selection medium containing hygromycin 10mg/L

B C

B. Elongated PKM-1 shoot on rooting medium; C. PKM-1 transformant withstanding in transgenic greenhouse

Fig-2 Agrobacterium mediated transformation of Tomato (cv. PKM-1) with pCAMBIA1300 carrying *EcNAC67*.

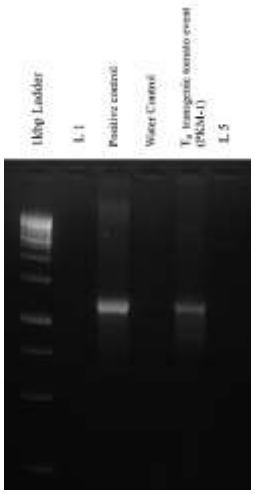


Fig-3 PCR analysis of putative transgenic tomato line(s) using gene specific primers of *EcNAC67*

Application of research: Developed transgenic plants will serve as a potential genetic material for unraveling molecular networks modulated by NAC67 transcription factor leading to enhanced drought/salinity tolerance.

Research Category: Agricultural Biotechnology

Abbreviations: PKM: Periyakulam; IBA: Indole-3- Butyric Acid

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Study area / Sample Collection: Department of Vegetable Crops, HC&RI, TNAU, Coimbatore

Cultivar / Variety / Breed name: Tomato (*Solanum lycopersicum* L.)

Conflict of Interest: None declared
Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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