

Agrobacterium-mediated transformation for development of transgenic tomato with *ySAMdc* gene

Prarthana Somayaji^{1,2}, Theertha Prasad Doddananappa² and Shivanna Manchanahally Byrappa^{1*}

¹Department of Applied Botany, Kuvempu University, Shankaraghatta, Shimoga 577 451, India

²Department of Biotechnology, University of Agricultural Sciences, GKVK, Bangalore 560 065, India

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Tomato is an important model plant system for transformation and expression of genes related to different characteristics. Enhancement of lycopene content in tomato fruit is one of the important aspects in improvement of commercial genotypes. In the present study, tomato genotype Arka Vikas was selected for introduction of 488 bp *ySAMdc* gene under E8 fruit promoter through transformed *Agrobacterium tumefaciens*. The transformation efficiency was 16.7 and 14.2% in two trials. This resulted in the production of 7 putative transgenics in T₀ generation and 4 in the T₁ generation. In both the generations, the lycopene content was enhanced by 2 to 3-fold. The transgenic plants expressed slightly altered morphological and biochemical characteristics. The integration of gene was confirmed using reverse transcription-PCR after extraction of RNA.

Keywords: *Agrobacterium*, BASTA, tomato, *ySAMdc*

Introduction

Agrobacterium-mediated gene transformation has been reported in different cultivars of tomato for a variety of purposes, which includes characterization of gene function, herbicide tolerance, disease and insect resistance, improvement in fruit quality, delay in fruit ripening and production of foreign proteins¹⁻³. Amongst several transformation techniques, *Agrobacterium* Ti plasmid vectors, microprojectile bombardment, microinjection, poly ethylene glycol-treatment and electroporation of protoplasts, *in planta* transformation using floral dip and agro-injection methods are most extensively used in plant transformation³.

A search of literature shows that tomato genotypes have been used for the expression of *ySAMdc* gene through *Agrobacterium*-mediated transformation and, particularly, those that are predominantly cultivated in USA and China^{1,4}. Although a large number of tomato genotypes are being cultivated in India, none of them have been taken for transformation studies involving *ySAMdc* gene. Previous studies revealed that tomato genotypes in India varied in their morphological, biochemical and ionic characteristics⁵. The genotypes grown in India had generally a low to

moderate lycopene content. Since lycopene is an important precursor of vitamin A and a very effective antioxidant, besides its role in enhancing post-harvest shelf life, experiments were designed to augment the lycopene content in one of the genotypes through *Agrobacterium*-mediated transformation of *ySAMdc* gene.

The objective of the present study was to standardize *Agrobacterium*-mediated transformation protocol using *ySAMdc* gene under E8 fruit promoter to produce putative tomato transgenics genetically enhanced in lycopene content. Morphological characteristics like plant height, number of flowers and fruits, fruit cluster and fruit weight, and biochemical characteristics like lycopene, total soluble solids (TSS) and titratable acid content of transgenic plants were also determined.

Materials and Methods

Selection of Tomato Genotype and Tissue Culture Media

Tomato seed sample of Arka Vikas was collected from Indian Institute of Horticultural Research, Hessarghatta (Bangalore). The seeds had 100% germinability. The tissue culture medium Murashige and Skoog (MS) (Himedia, Mumbai) was modified by amendment with antibiotics, plant growth hormones and selection markers, and categorized into MS-modified A, B, C, D, E and F (Table 1).

*Author for correspondence:
E-mail: mbshivanna@yahoo.co.uk

Selection of *Agrobacterium* Strain, Plasmid and *ySAMdc* Gene

The *ySAMdc* gene was isolated and cloned in Dr Handa's laboratory (<http://www.chromdb.org/plasmids/pFGC5941.html>). The plasmid used had a total size of 11406 bp and the gene size 488 bp. The plasmid contained 2 selection markers one for BAR gene resistant to BASTA (ammonium glufosinate, herbicide) and another plasmid *Km* marker resistant to kanamycin. The entire gene was cloned to pFGC5941 plasmid and was transferred into *Agrobacterium* strains.

Co-cultivation of Tomato Cotyledons with Transformed *Agrobacterium*

The seeds were subjected to germination after surface disinfection (ethanol 70%, 1 min; HgCl_2 0.01%, 3 min) on MS modified medium A. Cotyledons of tomato seedlings (10 to 12-d-old) were excised and transferred to the yeast extract peptone (YEP) (Himedia, Mumbai) broth containing *ySAMdc* harboured *Agrobacterium* and swirled for 5 min. They were removed and washed in SDW. After blotting, cotyledons were transferred to plates containing MS modified medium B containing 100 μM acetosyringone. The plates were incubated in dark for 24 h and then transferred to culture room (25°C, 95% RH, light intensity of 30 lux) and incubated further for a period of 24 h.

Callus Initiation and Plantlet Regeneration

After co-cultivation, cotyledons were washed, blotted and transferred with its adaxial side on the surface of the MS modified medium C. All explants categories were transferred separately to the fresh medium at an interval of 2 wk. Calli were cut into 1 cm^2 pieces and transferred to the same medium after treatment with cefotaxim (250 mg L^{-1}). After 4 wk of culture, calli were transferred to MS modified medium D, followed by MS modified medium E for another 2 wk. The calli not co-cultivated and not infected with *Agrobacterium* or BASTA treated served as positive control, and those not co-cultivated but infected with transformed *Agrobacterium* were used as negative control.

The 3-4 cm long shoots obtained from *Agrobacterium*-infected calli were excised under sterile conditions and transferred to rooting MS modified medium F. Plantlets with well developed roots were washed carefully under slow running tap water, treated with carbendazim solution (0.1%) for 15 min and transferred to paper cups (8 cm diam $\times$ 12 cm length) containing sterilized cocopeat and irrigated with sterile water. Plantlets were maintained at 25°C, 95% RH and light intensity of 30 lux. Transplants were irrigated with half-strength MS broth (without hormones and vitamin) once a wk. 15-d-old seedlings (15-20 cm length) were transplanted into large earthen pots (22 cm $\times$ 30 cm) containing potting mixture (farm yard manure, red soil and sand, 1:2:1 w/w) under isolation for 120 d. Morphological characteristics, such as, plant height, number of flowers and fruits, fruit wt and fruit cluster were determined. Biochemical traits, such as, total soluble solids (TSS), titratable acidity and lycopene were estimated in red ripe fruits of tomato.

Molecular Analysis of Primary Transformants of Tomato

The putative primary transformants (transformed or non-transformed with *ySAMdc* gene) of tomato obtained by *Agrobacterium*-mediated transformation method was subjected to PCR. The genomic DNA was isolated separately from young leaves (at flowering stage) of putative transformed and non-transformed plants by modified CTAB method^{6,7}. The DNA yield was quantified at 260 nm and the purity was calculated using 260/280 nm absorbance ratio⁸. The plasmid containing *ySAMdc* gene was isolated from *Agrobacterium* strain and integration of *ySAMdc* gene was confirmed by PCR analysis using the SAM-specific forward and reverse primers at 1.5% agarose gel.

Isolation of Plasmid DNA

A loop of transformed *Agrobacterium* colony culture was taken and added to 50 mL YEP broth with 6.25 μL kanamycin (50 $\mu\text{g mL}^{-1}$). This culture was

Table 1—Modifications of MS medium by specific amendments

Types	Constituents
MS Medium A	MS medium without hormones
MS Medium B (Co-cultivation medium)	MS medium+0.1 mg L^{-1} IAA+4 mg L^{-1} BAP+100 μM acetosyringone
MS modified C (Proliferation medium)	MS medium+0.1 mg L^{-1} IAA+4 mg L^{-1} BAP
MS modified D (Selection medium)	MS medium+0.1 mg L^{-1} IAA+2 mg L^{-1} BAP+2 mg L^{-1} BASTA
MS modified E (Selection medium)	MS medium+0.1 mg L^{-1} IAA+4 mg L^{-1} BAP+4 mg L^{-1} BASTA
MS modified F (Rooting media)	MS medium+0.5 mg L^{-1} IAA+0.5 mg L^{-1} BAP) medium

grown overnight to obtain an OD (600 nm) of 0.5, 0.8 and 1.0 and the efficiency were tested accordingly. The 1.5 mL broth culture was taken into sterile eppendorf tubes and centrifuged at 11269 g for 10 min at 4°C. Reagents suggested by Sambrook *et al*⁸ were used for isolation of pFGC5941 plasmid containing *ySAMdc* gene from *Agrobacterium* strain. The pellet was extracted with solutions I, II and III. The pure plasmid obtained by this procedure was stored at -20°C.

Expression of *ySAMdc* Gene in Transgenic Tomato Fruits Using RNA Technique

The fruits produced in transgenic progeny were harvested at red ripe stage and freeze dried using liquid nitrogen (-190°C). The RNA was extracted from these fruits by LiCl₂ method^{8,9} with certain modifications. Accordingly, 4 g of tomato fruit tissue was crushed using a DEPC (0.1%) treated pestle and mortar to obtain the extract, which was taken in a DEPC treated clean oak ridge tube. To this extract, 16 mL of extraction buffer (1.4 M NaCl, 100 mM Tris, 20 mM EDTA, 2% CTAB (w/v), 1% SDS, 2% β-mercaptoethanol, and 4% PVP, at 65°C), and 13 mL mixture of phenol-chloroform-isoamyl alcohol (25:24:1) was added. Two additional treatments with 3 M and 2 M lithium chloride were also given before final washing with 70% ethanol. The purity of RNA was quantified using 260/280 nm absorbance ratio. Total RNA from the transgenic tomato fruit (freeze dried) was electrophoresed on the denaturing formaldehyde agarose gel. First strand cDNA synthesis was carried out by reverse transcribing total RNA using RevertAidTM M-MuLV reverse transcriptase. The size of the cloned DNA fragments (488 bp) was determined by using DNA ladder/marker (0.5-5 Kb). Integration of *ySAMdc* gene was confirmed by PCR analysis [total reaction mixture (20 µL) consisted of: DDMW, 8.2 µL; 1× *Taq* assay buffer, 2.0 µL; 200 µM dNTPs, 4.0 µL; 1.5 mM MgCl₂, 1.2 µL; 10 ng µL⁻¹ Template DNA, 2.0 µL; *Taq* DNA polymerase, 1.5 U] using *ySAMdc* specific forward and reverse primer (10 pmol, 2.5 µL).

To validate the molecular transformation, the transgenic and control plants were subjected to swab tests. Healthy leaves of the above categories of plants were selected, tagged and swabbed with 0.5, 2, or 4 mg L⁻¹ of BASTA on the abaxial and adaxial leaves surfaces. The seeds collected from the fruits of T₀ lines grown in pots. The T₁ transgenic lines were studied for their morphological (plant height, number of flowers and fruits, fruit cluster & fruit wt) and biochemical (TSS, lycopene & titratable acidity) characteristics.

Results

Transformation Efficiency and Plantlet Regeneration

Agrobacterium-mediated transformation of *ySAMdc* gene in tomato resulted in 16.7 and 14.2% transformation efficiency (average 15.4%) in two trials (Table 2). The pin-pricked cotyledons were explants infected with *Agrobacterium* for 3 min. The co-cultivation accentuated the efficiency of transformation in the presence of 100 µM acetosyringone. All the explants (co-cultivated and not co-cultivated with *Agrobacterium*) produced good callus cultures within 3 wk. The callus obtained was further transferred to MS modified medium D containing 2 mg L⁻¹ BASTA. The normally grown calli and shoot primordia were then transferred to MS modified medium E containing 4 mg L⁻¹ BASTA. In this study, two treatments of BASTA was given because BASTA is a powerful herbicide and according to the studies conducted previously, concentration above was deleterious and caused senescence. Calli that survived under stringent condition was excised and re-cultured on MS modified medium C. Then developed shoots (3 cm length) were placed on the MS modified medium F (rooting medium), which produced 100% rooting. Shoots obtained from positive control (not co-cultivated and not infected with *Agrobacterium* or BASTA treated), *i.e.*, untransformed explants, produced good roots. In negative control (not co-cultivated but infected with transformed *Agrobacterium*), calli failed to regenerate any shoot and started senescing. The

Table 2—Efficiency of *Agrobacterium*-mediated tomato transformation

Trail	Explants infected (no.)	Explants survived on BASTA medium (no.)	Explants regenerated (no.)	Transformation efficiency (%)
1	60	19	10	16.7
2	70	23	13	14.2
Average	65	21	11.5	15.4

survival rate of rooted transgenic plantlets on the potting medium was 100%; however, only 80% of them survived when transferred to the greenhouse. All rooted positive control plants survived in the greenhouse.

Molecular Screening of Transgenic Tomato Plants Containing *ySAMdc* Gene

Genomic DNA amplification using SAM specific primers produced DNA fragment of 488 bp, however no amplification was observed in negative control (non-transformed plant). Out of the 16 transformed plants, only 7 putative transgenic plant lines (AVT2, AVT3, AVT9, AVT6, AVK11, AVT13 and AVT15) produced desired *ySAMdc* gene band of 488 bp (Fig. 1a). Further, the RNA extracted from ripened fruits of transgenic plants confirmed the transformation when tested on formaldehyde gel (Fig. 2a). The cDNA synthesized using *ySAMdc*-specific primers also confirmed the presence of SAM gene of 488 bp (Fig. 2b).

Seven tomato transformants (T_0 generation) were exhibited normal and healthy plant growth. Among transgenic as well as control plants, control plants fared better in morphological traits. Biochemical tests indicated 2-3 fold increase in lycopene content of transformed fruits as compared to that of the

non-transformed, suggesting the efficient insertion of *ySAMdc* gene. Also, a significant increase in the titratable acidity and TSS was noticed in transgenic fruits as compared to the controls (Figs 3 & 4).

T_0 transgenic plants were further grown and young leaves and ripened tomato fruits were collected. PCR analysis of DNA from young healthy leaves

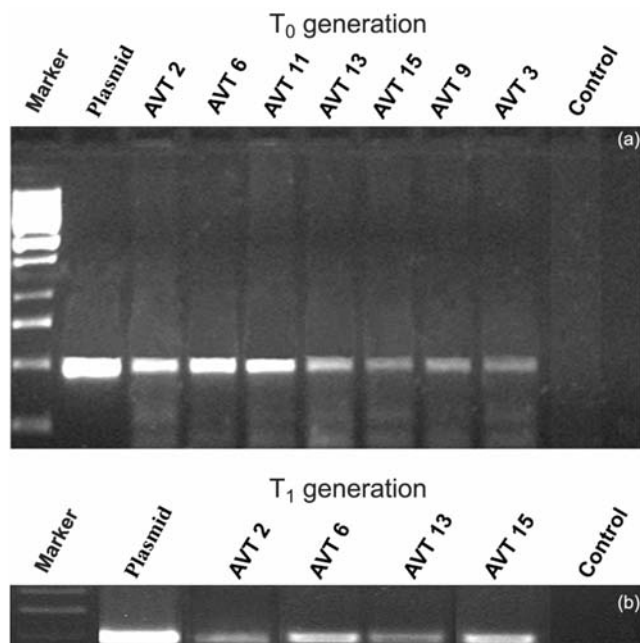


Fig. 1 (a & b)—(a). PCR analysis of 7 transgenic tomato plants (T_0 generation) for the presence of *ySAMdc* gene (488 bp). The marker lane represents 0.5-5 kb ladder. (b). PCR analysis of 4 transgenic tomato plants of T_1 generation showing the presence of *ySAMdc* gene (488 bp). [The amplified products were resolved in 1.5% agarose gel]

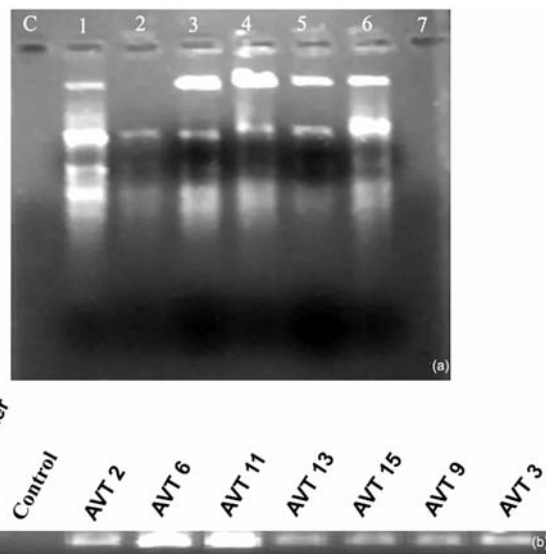


Fig. 2 (a & b)—(a). Total RNA extracted from freezed dry fruits of 7 transgenic lines using $LiCl_2$ method. The RNA extraction was confirmed in formaldehyde gel. [C = Control; Lanes 1, 2, 3, 4, 5, 6 & 7 = Transgenic lines] (b). RT-PCR analysis of cDNA copies using gene specific primers to find out the expression of *ySAMdc* into the fruits.

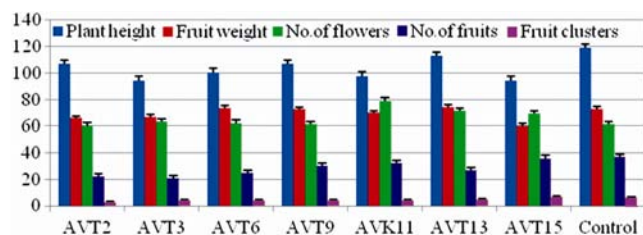


Fig. 3—Morphological characteristics like plant height (cm), fruit wt (g), number of flowers and fruits, and fruit clusters in T_0 transgenic tomato lines.

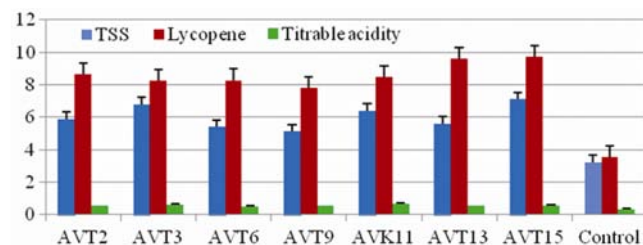


Fig. 4—Biochemical characteristics like total soluble solids (TSS) ($^{\circ}Bx$), lycopene ($mg\ 100\ g^{-1}$) and titratable acidity ($mg\ 100\ mL^{-1}$) in T_0 tomato transgenic lines.

(4 transgenic plants AVT2, AVT6, AVT13 & AVT15) showed the presence of 488 bp bands (Fig. 1b). A significant difference was observed in morphological and biochemical characteristics of control and transgenic lines of T₁ generation grown in the greenhouse (Figs 5 & 6).

Swab Tests for Confirmation in T₁ Generation

Leaves of non-transformed plants produced necrosis following the application of BASTA. Concentration of 2 mg L⁻¹ BASTA showed partial browning, while 4 mg L⁻¹ showed complete necrosis of leaves, suggesting that the plant was not transgenic. However, leaves of transgenic plants remain unaffected to both the concentrations of BASTA.

Discussion

Tomato is highly acquiescent to physiological and cytological studies due to the ease in culture and genetic uniformity resulting from autogamy¹⁰. Genetic manipulation of carotenoid biosynthesis in higher plants, especially the accumulation of lycopene in tomato fruits, has attracted the attention of many researchers. The studies of tomato and citrus as model plants for pigmentation mutants¹¹ and transgenics¹² have provided insights into the regulatory mechanism(s) operating in carotenogenesis. Some imperative changes that occur are the production of aromatic compounds and nutrients, colour and softening of the flesh to an edible texture, which have evolved to attract animals and promote seed dispersal¹³. During the process of ripening, levels of spermidine and spermine decline in fruits. Transgenic tomato plants expressing yeast *SAMdc* gene fused to the ripening-specific promoter E8 over-accumulated spermidine and spermine at the cost of putriscene¹.

The plant transformation by *A. tumefaciens* has become the most accepted method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants because of its simplicity and efficiency. Transformation efficiency depended on various factors, such as, the cultivar, type and age of explant, *Agrobacterium* strain and its density, co-cultivation period and time, effect of acetosyringone, and dosage of cefotaxim¹⁴. Tomato genotype Arka Vikas, a product from Indian Institute of Horticulture Reserch, Hessarghatta, Bangalore, exhibited an average response to different morphological, biochemical and ionic characteristics⁵. Earlier, a study was conducted to test

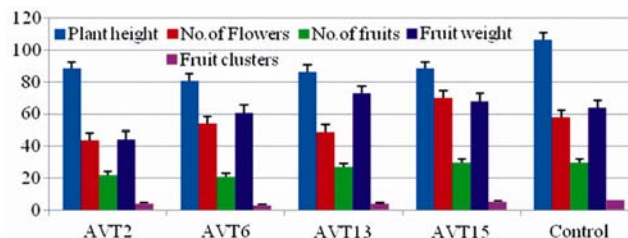


Fig. 5—Morphological characteristics like plant height (cm), fruit weight (g), number of flowers and fruits, and fruit clusters in T₁ transgenic tomato lines.

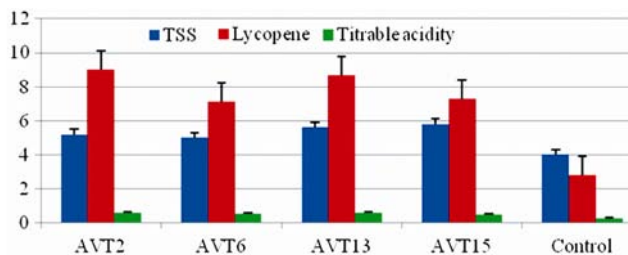


Fig. 6—Biochemical characteristics like TSS (°Bx), lycopene (mg 100 g⁻¹) and titratable acidity (mg 100 mL⁻¹) in T₁ transgenic tomato lines.

the transformation efficiency in Arka Vikas by incorporation of *nptII* as selectable marker and *GUS* as reporter gene¹⁵. However, fruit quality of the above transgenic genotype was not evaluated. Hence, genotype Arka Vikas was selected in the present study for introducing *ySAMdc* gene via *Agrobacterium*-mediated transformation.

In the present study, different concentrations of *Agrobacterium* (0.5, 0.8 or 1.0) were tested. Amongst which the concentration of 0.8 at an OD of 600 nm produced optimum results upon co-cultivation of cotyledon explants with bacterial cells at 48 h of incubation. However, the transformation efficiency varied with respect to time of co-cultivation. Explants co-cultivated for 1 min senesced on selection media as there was hardly any bacterial infection in them, while explants co-cultivated for more than 5 min caused excessive growth of *Agrobacterium* and resulted in reduced transformation efficiency. The explants cultured for 3 min showed good transformation efficiency and found in accordance with studies of Jabeen *et al*¹⁴. In the present study, an average of 15.4% transformation efficiency was achieved by *Agrobacterium* transformation and these results corroborated with the earlier findings on genetic transformation protocol developed using *ySAMdc* gene¹.

Further, 100 µM acetosyringone induced good transformation efficiency. However, in another study,

50 μ M acetosyringone was reported to induce optimum transformation efficiency in tomato cv. Lichan¹⁴. Higher concentration of acetosyringone was found less effective¹⁶. For the expression of pFGC5941 plasmid DNA, kanamycin was used as a selective marker. In the present study, 50 mgL^{-1} of kanamycin was found effective in the agrobacterial transformation, which is in accordance to Sarker *et al*¹⁵.

BASTA (glufosinate) was used as a selection marker for *ySAMdc* gene in the selection of transformants at very low concentration of 2 and 4 mgL^{-1} . Jones *et al*¹⁷ has also reported the use of glufosinate as selection marker of bar gene in the transformation of wheat. It is reported that under conditions of non-recommended usage of glufosinate, a detrimental effect on the health of both users and consumers is extremely unlikely¹⁸.

The parameters of morphological characteristics (plant height, fruit number, fruit wt) of *Agrobacterium*-mediated *ySAMdc* transgenic plants were less as compared to control plants and reduction in fruit wt may be attributed to high levels of metabolism and decrease in sugar content. However, lycopene content, TSS and titratable acidity in transgenic fruits of T₀ and T₁ generations were comparatively high as compared to fruits of non-transgenic lines (Figs 3-6). These results are in accordance with previous studies^{1,4,27}.

Conclusion

The results of *Agrobacterium*-mediated transformation of *ySAMdc* gene in tomato resulted in an average 15.4% transformation efficiency in two trials and genomic DNA contained 488 bp insert of SAM gene. The putative transgenics showed significant increase in lycopene and TSS contents and high titratable acidity levels as compared to control. Morphological and biochemical observations showed no drastic changes in T₁ generation as compared to T₀ generation. Further studies relating to gene sequencing, analysis of gene expression, variation, protein profiling work and observation of the physiological changes over generations as well as validation of gene expression in T-2 and T-3 generations of tomato transgenics for segregation patterns, stability and expression of the desired gene of interest are required for a complete understanding.

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