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ORIGINAL ARTICLE

Enhancement of Chlorogenic Content of the Eggplant Fruit with Eggplant Hydroxycinnamoyl CoA-Quinate Transferase Gene via Novel Agroinfiltration Protocol

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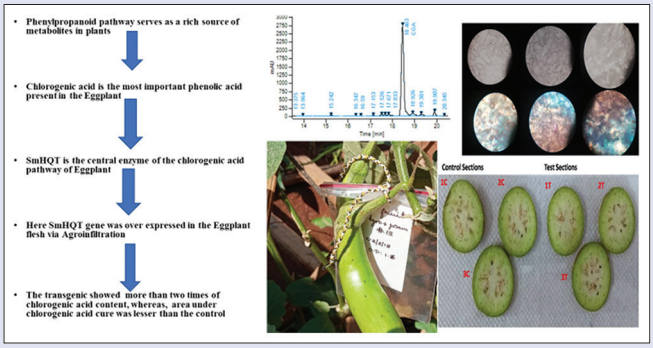
ABSTRACT

Background: Eggplant (*Solanum melongena* L.) is rich in health-promoting phenolic acids, primarily in the chlorogenic acid. For the production of chlorogenic acid in the eggplant hydroxycinnamoyl CoA-quinate transferase (SmHQT), is a central enzyme that catalyzes the reaction to the chlorogenic acid production. **Objective:** The function of SmHQT is not well determined in the eggplant fruit, and the fruit agroinfiltration procedure is not standardized for eggplant. **Materials and Methods:** Here, the overexpression of SmHQT in the eggplant fruit´s flesh was studied using the agroinfiltration technique. In our gene construct, we also co-expressed the P19 protein for overexpression, and the results were validated with real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and high-performance liquid chromatography (HPLC). **Results:** Due to the overexpression of the SmHQT gene, higher chlorogenic content was exhibited by the eggplant fruits, which was further validated by HPLC. The chlorogenic acid content after following the agroinfiltration procedure was more two times in the agroinfiltrated fruit. To identify the optimal target for increasing chlorogenic pathway flux post-SmHQT activity, expression patterns were analyzed with qRT-PCR, and the results showed the changes in the expression level of the other chlorogenic acid pathway genes. Furthermore, the cis regulating elements and protein-protein interaction (PPI) analyses supported the HPLC results. **Conclusion:** Overall, here, insights into the eggplant chlorogenic content increment at the molecular level and the opportunities for the improvement of chlorogenic content as nutrition in crops are provided.

Key words: Agrobacterium, agroinfiltration, chlorogenic acid, eggplant, eggplant hydroxycinnamoyl CoA-quinate transferase, transcriptome

SUMMARY

- In a nutshell, we have created an agroinfiltration protocol for the transient expression of a gene inside the eggplant fruit and employing this protocol, we have overexpressed the eggplant hydroxycinnamoyl CoA-quinate transferase, which is also the central enzyme studied to enhance the chlorogenic acid content. Also, in our cassette, we co-expressed the P19 protein of Tomato bushy stunt virus to overexpress the protein. This has resulted inside the doubling on the chlorogenic acid content material inside the Eggplant fruit. All round, we hope this data are going to be valuable in reaching a profitable eggplant ideotype.



Abbreviations used: 4CL: 4-hydroxycinnamoyl-CoA Ligase; C3H: p-coumaroyl ester 3′-hydroxilase; C4H: Cinnamate 4-hydroxilase; CGA: Chlorogenic acid; DAI: Days after infiltration; HPLC: High-performance liquid chromatography; OE: Overexpression; PAL: Phenylalanine ammonia-lyase; PPI: Protein-protein interaction; PTGS: Posttranscriptional gene silencing; qRT-PCR: Real-time quantitative reverse transcription PCR; SmHQT: Eggplant hydroxycinnamoyl CoA-quinate transferase.

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INTRODUCTION

Phenolic acids are widespread among the plant kingdom.[1,2] Among vegetables, phenolic acids, for example, chlorogenic acid, is present in larger quantities in the eggplant (*Solanum melongena* L.). Moreover, the phenolic acids present in eggplant and its wild relatives have proved valuable for the protection against many ailments such as diabetes, cancer, and arthritis.[3-5] Therefore, enhancing the content, of these health-promoting phenolic compounds, especially chlorogenic acid, is among the important breeding objectives for eggplant. The chlorogenic acid (5-caffeoylquinic acid) is around 90% of total phenolics present in the eggplant fruit.[6-8] For plant, chlorogenic acid aids in protection against insect pests and pathogen infestations.[9,10] The cultivated eggplant has far significantly less phenolic acids than its many wild relatives.[5] Therefore, many breeding strategies were undertaken to introgress the genes for higher fruit phenolics to the cultivated eggplant from its wild relatives.[11] However, all these tactics have resulted in limited success.[2,12] Moreover, the genome editing approaches and transgenic technologies cannot be overlooked.[13-15]

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The Agrobacterium-mediated transformation is among one of the most common methods of plant transformations.[16-18] In this direction, the agroinfiltration strategy for the transient expression of a gene is also regularly applied to determine the function of the gene.[19] In plants, there is certainly a large prospective to mass-produce recombinant proteins (e.g., enzymes).[20-22] Furthermore, the transient protein expression is useful to provide desired information about the gene function in days as compared to the other approaches.[23] This strategy is properly established in many fruit-bearing plants such as tomato, strawberry, melon, and cucumber.[24-26]

The chlorogenic acid synthesis pathway is identified in eggplant along with the enzymes catalyzing the different steps of the pathway.[27] Whereas the function of the eggplant hydroxycinnamoyl CoA-quinate transferase (SmHQT) is not studied in detail as compared to its homolog in Tomato and Potato.[28-32] Therefore, the objectives of this study have been to establish and standardize an efficient agroinfiltration protocol for the eggplant fruit. In our transgene cassette, we also co-expressed the P19 protein gene of tomato bushy stunt virus, to prevent the posttranscriptional gene silencing.[33] SmHQT was overexpressed and interactome analysis and high-performance liquid chromatography (HPLC) of the same was performed to understand network underlying and the effect in fruit.

MATERIALS AND METHODS

Plant material

The Eggplant seeds of variety Arka Shrish a popular green fruited eggplant cultivar released by IIHR (Indian Institute of Horticulture Research), India, were grown using soil and perlite (2:1) in the presence of natural light at 20°C–25°C.

In silico cis-regulating elements map and interactome analysis

The genomic sequences of SmHQT (hydroxycinnamoyl CoA-quinate transferase) genes were retrieved from the http://eggplant.kazusa.or.jp. They were processed through PLACE software for the determination of the binding sites using up to 10000 bp upstream sequences.[34,35]

Development of the SmHQT gene construct with the specific promoter in a plant transformation vector.

Genomic DNA was extracted from the fruits and was amplified for the SmHQT gene. Later cloned within a pUC cloning vector (pBlueScript) and sequencing was performed for the confirmation. Constructive clones were confirmed and processed for Sub-Cloning using the expression vector (pBIN19, Addgene). Further, the gene was cloned inside the cloning vector (pBlueScript KS + vector). The pBS + SmHQT clone was restriction digested (HindIII/BamHI). The SmHQT gene was ultimately sequence confirmed and utilized for agroinfiltration assays. First, we have used the GUS bearing vector pCAMBIA1304 (Adgene) for the standardization of the eggplant fruit agroinfiltration protocol. The culture was sub-cultured using LB broth (5 ml) and at an O. D of 1.6 was used for the agroinfiltration using a 2 ml syringe was injected into the Eggplant fruits at 10–15 spots and permitted to develop for 3–10 days soon after infiltration (DAI). The HPLC analysis was performed based as described previously5, and the qRT-PCR analysis was performed as defined elsewhere.[36]

RESULTS

In silico cis-regulating elements analysis

A 1.0 kb sequence upstream to open the reading frame of the SmHQT gene was taken for analysis. In general, the analysis suggests that the presence of multiple numbers of responsive elements for stress and hormonal regulation elements were identified. Based on their location in the upstream gene region, the cis-regulatory elements were mapped using online programming mentioned in the material method. The results showed that most of the cis-regulating elements belong to the activation mechanism of GT-1.

Meanwhile, the results suggested that stress-responsive and light-responsive elements were present in the HQT genes. Similarly, the remaining cis regulating elements, most of them are hormonal regulation responsive elements. This may lead to the hypothesis that the transcript occurrence of this HQT gene and elements could be easily affected by the presence of light responses and other hormones regulation, i.e., CACTFTPPCA1 and CCAAT.

Interactome analysis

In the current study, the function of SmHQT genes was predicted *in silico*, by employing Arabidopsis homologs. Protein-protein interaction (PPI) used initial premilarly screening of interacting patterns of the candidate genes. In this study, the PPI interactome analysis results suggest that the HQT genes have many interacting partners such as TT4, CYP98A3, 4CL3, 4CL1, C4H, IRX4, 4CL2, LysoPL2, and CCOAMT [Figure 1]. Most of the interacting patterns are belonged to the phenylpropanoid pathways, by manipulating these patterns may lead to upregulation or downregulation of the candidate genes which may directly affect the end product. If the targeted genes were overexpressed or knockdown, these pathways patterns are also affected [Figure 1].

Standardization of agroinfiltration protocol and overexpression of the SmHQT gene after 3 days following agroinfiltration assay fruit samples were harvested by employing the GUS gene-based X-Gluc staining method. Further, it was conformed that the fruits samples 3 DAI showed the best results compared to the samples 7 DAI and 10 DAI.

The coding sequences of SmHQT were cloned with PCR-based methods. The mRNA of SmHQT (AMK01803.1) encodes a protein with a peptide sequence of 427 amino acids. The gene was overexpressed under the control of CaMV35S promoter SmHQT. Similarly, transient expression was also done with the native promoter [Figure 2a]. Among the 20 fruits overexpressing SmHQT, fruits were determined with phenotypic changes as compared to the control plants [Figure S1]. The Agrobacterium tumefaciens GV3101 strain was used and transformed

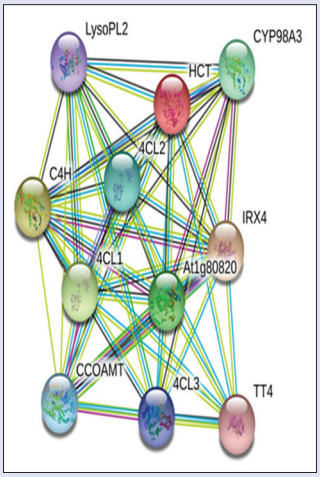

Figure 1: Protein-protein interaction networks SmHQT controlling high chlorogenic biosynthesis pathway in eggplant using Arabidopsis databases. Their interactions were analysed online using STRING database (https://string-db.org/)

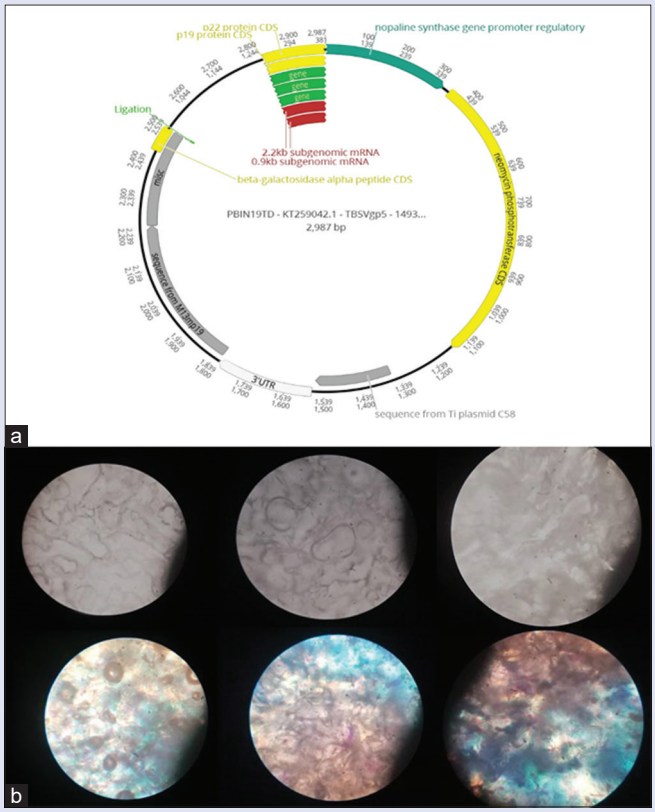

Figure 2: Construct used for the agroinfiltration assay (a). A comparison of fruit slices after following the X-Gluc staining procedure with the control fruit slices are above (b)

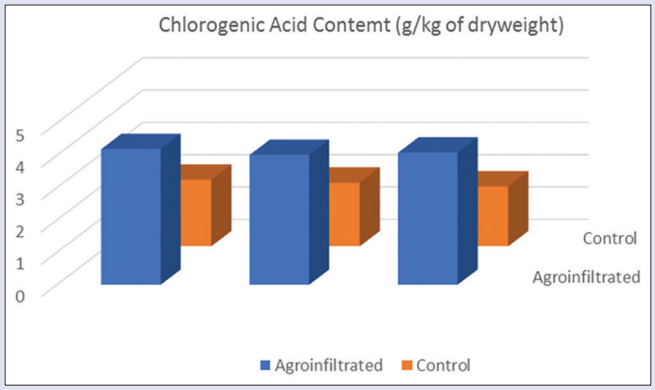

Figure 3: The high performance liquid chromatography results of agroinfiltrated versus control fruits in three independent fruits on different plants

to, GUS bearing vector with SmHQT was transiently expressed in eggplant fruit [Figure 2b].

High-performance liquid chromatography analysis

The amount of chlorogenic acid content was estimated with the help of calibration curves [Figure 3]. The results of the HPLC analysis are presented in Figure 3. The transgenic showed more than two times of chlorogenic acid content, whereas the area under the chlorogenic acid curve was lesser than the control [Figure 3].

Quantitative gene expression analysis

Expression analysis of phenylpropanoid chlorogenic biosynthetic genes targeted were those active in the early stage of the pathway, i.e., PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxilase; and 4CL, 4-hydroxycinnamoyl-CoA ligase [Table S1]. Furthermore, the late phase genes of the chlorogenic acid pathway, namely, DFR, dehydroflavonol reductase, and C3H, p-coumaroyl ester 3-hydroxylase; were also checked. On an average, the relative expression levels were upregulated in agroinfiltrated fruits, were of PAL, C3H, and SmHQT; their transcripts were elevated in 1 and 3 days after infection experiments, in which transcript level was high almost greater than six-fold and sevenfold respectively. The relative expression levels were several folds higher in the agroinfiltrated fruits as compared to the control sample [Figure 4].

DISCUSSION

Chlorogenic acid has an advance property to human make food cravings, reduces daily calorie intake and induces body fat and also discharges glucose into the bloodstream.[37] In this study, nine potential protein interaction networks (TT4, CYP98A3, 4CL3, 4CL1, C4H, IRX4, 4CL2, LysoPL2, and CCOAMT) were identified for HQT gene.

The role of the isolated SmHQT in the regulation of the phenylpropanoid biosynthetic pathway was investigated through a transient transformation in eggplant to study the functional role of the SmHQT with its native promoter.[30,38] The vector pBIN19 + SmHQT, transfected into Agrobacterium and infiltrated into eggplants fruit, alongside with the empty vector. Five days postinoculation, SmHQT agro-infiltrated eggplant fruit showed an anthocyanin-pigmented phenotype, in control type eggplants. For more conformation, HPLC and gene expression were also performed. The overexpression of SmHQT, hydroxycinnamoyl CoA quinate transferase (HQT) is the critical enzyme catalyzing CGA biosynthesis in *S. melongena*. Transgenic *S. melongena* plants that overexpress HQT (OE) fruiting from original transformants were used in this study. Almost all gymnosperm and angiosperm contain three valuable content like first is sinks for photosynthetically fixed carbon second is for cellulose/hemicellulose (cell walls) and finally third one starch, TAG and Phenolic compound. This relative content varies according to the system also depends on the developmental stage and plant species.[39] These entire phenolics compounds produced by plants are increasingly relevant for various biotechnological and pharmaceutical uses.[40,41]

The gene expression depends upon the transcriptional regulation of that gene; it depends on on co-expressed genes a gene signature and motifs present.[42,43] Most of the time, TFs connecting to this motif; many direct affect the interactions and ends with up and downregulation of that gene. When in the promoter region, more the motifs availability more the chance for upregulation.[44] However, the application of the same methods to higher eukaryotes has not been fruitful, not even too small sequence search spaces, for example, promoters,[45] so it essential to identify minimal promoter region for that TFs and gene. Although we find putative sites for various transcriptional regulation by multiple factors in HQT genes, most of the sites in promoter region responsible for hormonal control and light regulation which required further functional validation, the abundance of cis regulating elements like GT-1.

PPI interactome analysis also supports that promoter analysis (cis regulating elements) results suggest that most are the phenolic compound are interconnected, and the pathways are interdependent, like TT4.[46] CYP98A3, related to cytochrome P450, family, subfamily, cytochrome P450, which catalyzes hydroxylation of p-coumaric esters of shikimic/quinic acids to form lignin monomers. In arabidopsis At1 g80820, Cinnamoyl-CoA reductase 2 reductases probably involved in

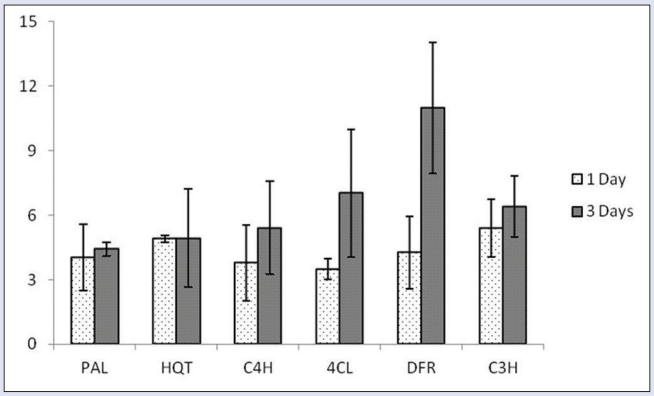

Figure 4: Differential gene expression level (quantitative reverse transcription polymerase chain reaction) of six candidate genes of the chlorogenic pathway is represented with respect to control plant fruit. The expression was analysed at one day and 3 days after infection. Data is presented as mean ± standard deviation

the formation of phenolic compounds.[47,48] Similarly, a recent study shows that SmHQT improves other agronomical traits even in tomato. However, a network and cis regulating elements analysis suggest that the understand the regulation of SmHQT to the targeted chlorogenic pathway and also open the door whether play function similarly in other plant species.

CONCLUSION

Overall, an agroinfiltration protocol for the transient expression of a gene inside the eggplant and employing this protocol we've got overexpressed the SmHQT, which can be the central enzyme studied to enhance the chlorogenic acid content material, within a gene construct together with the distinct promoter within a plant transformation vector (pBIN19). Furthermore, in our cassette, we co-expressed the P19 protein of Tomato bushy stunt virus to overexpress the protein. This has resulted inside the doubling on the chlorogenic acid content material inside the Eggplant fruit. Further, we hope this data is going to be valuable in reaching a profitable eggplant ideotype.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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Table S1: Primer list Nucleotide sequences of primer pairs designed for the selected chlorogenic pathway in eggplant for real-time quantitative gene expression analysis (real-time quantitative reverse transcription - polymerase chain reaction)

Gene	F Prmers	Tm	R Primers	Tm	Accession number
PAL	TCGCTATGCTCTCCGAACAT	58.77	AGCTCCGAAAATTGGGCAAA	58.97	FS058603.1
SmHQT	ATCTCAACCTTCCCACTCGT	57.45	GGAGGGTCCGATCGATGAAT	58.37	FS083932.1
C4H	TGAGGCTCAACAGAAGGGAG	56.34	AAGCTTGTAGGTGTCTGGCT	55,00	FS082784.1
4CL	CCGGATACGGGTTGCTCTC	58,76	CCGGCGTGTAACCATCCT	58.34	FS021677.1
DFR	AGGACCCTGAGAATGGAGTAA	55.34	TCAAGAGTTCCAGCAGATGAAG	56.55	FS074352.1
C3H	GAATGGACACAACTGCAATCTCT	55.67	GGAAGTCTGTTTCGTTCATCACA	56.33	FS013298.1

PAL: Phenylalanine Ammonia Lyase; SmHQT: Eggplant Hydroxycinnamoyl CoA-quinate Transferase; C4H: Cinnamate 4-hydroxilase; 4CL: 4-hydroxycinnamoyl-CoA Ligase; DFR: Dehydroflavonol reductase; C3H: p-coumaroyl ester 3'-hydroxilase