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SHORT COMMUNICATION

Overexpression of Pepper Capsaicinoid Pathway Genes in Tomato (*Solanum lycopersicum* L.)

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ABSTRACT

Objective: The Solanaceae family includes many unique and popular fruits and vegetables such as potato, tomato, and pepper. Peppers are a group of plants that produce pungent fruits favoured by many in various parts of the world. This spiciness is due to a class of compounds called capsaicinoid which are synthesized in peppers but not in tomatoes. Both pepper and tomato genomes have been sequenced, and genes involved in the capsaicinoid biosynthesis pathway have been identified in both genomes. Along with expression profiling, there were only three genes in the tomato pathway that were not expressed. In this study, we attempted to overexpress the three pepper genes in tomato to produce spicy fruits.

Materials and Methods: The three genes, *BCAT* (branched-chain amino acid aminotransferase), *Kas* (ketoacyl-ACP synthase), and *CS/AT* (capsaicin synthase/acyltransferase), were separated using P2Am and T2Am sequences in a tricistronic cassette driven by the 35S promoter. Transgenic tomato plants containing the gene construct were generated via *Agrobacterium*-mediated transformation.

Results: RT-PCR indicated that the genes were expressed in all transgenic tomato plants. Some transgenic fruits resembled hot peppers with elongated shapes and wrinkled surfaces, but tomato fruits were not spicy based on two-person tasting evaluations.

Conclusion: P2Am and T2Am sequences can be used for the overexpression of multiple genes in tomatoes. Further studies with tissue-specific promoters and metabolic profiling are necessary.

Keywords: Capsaicinoid pathway genes, Overexpression, Pepper, Tomato

INTRODUCTION

The pungency of hot peppers (*Capsicum ssp.*) is the accumulation of a group of alkaloids called capsaicinoid, such as capsaicin and dihydrocapsaicin.[1] Capsaicinoids are synthesised in pepper fruit placenta through two biochemical pathways. In the phenylpropanoid pathway, phenylalanine is converted to vanillylamine, and in the branched chain fatty acid pathway, valine is converted to 8-methyl nonenoic acid.[2] Capsaicin synthase (CS) then combines both compounds to produce capsaicin and other capsaicinoids.[3]

Pepper is a common spice used in varieties of cuisine all over the world and is an excellent source of vitamins C, A, B-complex, and E.[4] As a medicine, it is a counter irritant in lumbago, neuralgia, rheumatic disorder, and non-allergic rhinitis and as a folk remedy for dropsy, colic, diarrhoea, asthma, arthritis, muscle cramps, and toothache.[5,6] These findings suggest that a diet with capsaicinoid is beneficial for health and thus hot peppers should be considered a functional food.[7] Peppers are often cultivated in open fields and are vulnerable to environmental conditions that are detrimental to fruit yield.[2] For example, high temperature, high CO_2 level, and excess rain can all reduce plant growth and fruit yield and increase disease incidence in the plants.[8] Seed germination rate highly depends on fruit maturity, species, cultivar, and post-harvest handling.[9] In addition, environmental factors heavily influence capsaicinoid biosynthesis and consequently pungency levels.[10]

Plant breeding has significantly contributed to the development of new varieties.[11] Other genetic manipulations such as *Agrobacterium*-mediated genetic transformation are not trivial for peppers. Currently available pepper transformation protocols have low efficiency, poor reproducibility, and high genotype-dependence.[12,13] Engineering tomatoes to produce capsaicinoid represents an alternative.[14]

Tomato (*Solanum lycopersicum*) is the most important horticultural crop.[15] Despite their divergence 19 million years ago[16], the genomes of pepper and tomato are significantly conserved,

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with a basic chromosome number of x = 12 in both species and major conserved syntenic segments between them.[17] Tomatoes are more amenable to genetic transformation, are highly productive with a short cropping cycle, and can be used to produce capsaicinoids.[18,19] Phylogenetic analysis of gene families involved in capsaicinoid biosynthesis in pepper and their orthologs in tomato, potato, and *Arabidopsis* identified 51 gene families, of which 13 had independent pepper-specific duplication (such as *ACLd, AT3, b-CT, C3H, CAD, CCR, Kas I* and *PAL* genes).[17] Comparative transcriptome analysis identified several genes in the capsaicin biosynthesis pathway with different expressions between pepper and tomato fruits. At the fruiting stage *BCAT* (branched-chain amino acid aminotransferase), *Kas* (ketoacyl-ACP synthase), and *CS/AT* (capsaicin synthase/acyltransferase) are highly expressed in pepper fruit placenta but not in the tomatoes.[3] Comparative expression studies in non-pungent peppers vs pungent pepper also showed large deletions in the *CS* gene, which cause no or very low expression in non-pungent pepper.[20] These results indicate that changes in the expression of *BCAT, Kas*, and *CS/AT* enabled capsaicinoid synthesis in hot pepper fruits.[3,20] Therefore, pungency in pepper is under transcriptional control, and higher expression of capsaicinoid biosynthesis genes in the placental septum increases pungent levels.[21,22] Compared with peppers, some genes in tomatoes have lower levels of expression (*PAL, C4H, ACL* and *AMT*), others have lower levels of expression with temporally restricted expression (*COMT* and *FaTA*), and some are not expressed at all (*Kas, BCAT* and *CS*).[3]

Based on the genetic information on both pepper and tomato, it is theoretically possible to activate the capsaicinoid pathway in tomato. Three genome engineering strategies can be employed for this purpose. One is to use transcriptional activators like effectors (TALEs) [23] or CRISPR/Cas9[24] for multiplex activation of genes in pepper. The second strategy is the use of targeted promoter replacement through genome engineering for the activation of inactive genes in tomatoes.[25,26] The third involves the overexpression of the pepper genes (*Kas, BCAT* and *CS*) in tomato through *Agrobacterium*-mediated transformation. This study has used *Agrobacterium*-mediated tomato transformation to insert and overexpress three pepper genes involved in capsaicin biosynthesis: *BCAT, Kas*, and *CS/AT*. Two different vectors were prepared: one with the 35s promoter and the other with the fruit-specific E8 promoter. All three genes were included in a single tricistronic vector for transformation. All three genes are involved in capsaicin biosynthesis through the branched chain fatty acid pathway. *BCAT* condenses valine to alpha ketoisovalerate. *Kas* along with *ACL* (acyl carrier protein) is involved in fatty acid synthesis through isobutyryl-CoA to 8-methyl-6-nonenoic acid. CS/AT/PUN1 condenses vanillylamine from the phenylpropanoid pathway with 8-methyl-6-noneoyl-CoA from the branched chain fatty acid pathway to synthesize capsaicin.[3]

MATERIALS AND METHODS

Preparation of the Overexpression Gene Construct

The pCAMBIA1301 vector was used to prepare the overexpression gene construct. The tricistronic cassette with the three pepper genes was arranged as shown in Figure 1A. The three genes were separated using P2Ap and T2Ap peptides (Figure 1B) from Osborn et al. [27]. The peptides were reverse translated to DNA sequences (P2A and T2A in Figure 1B) and optimized for plants (*Arabidopsis*) using JCat [28] to produce the P2Am and T2Am (Figure 1B) used in Figure 1A. The cassette (Figure 1C) was synthesized by BioBasic (Amherst, NY, USA) and ligated into pCAMBIA1301 after digestion with *Nco*I and *Bst*EII. The construct was used to transform *Agrobacterium tumefaciens* strain LBA4404, and positive clones were used for tomato transformation (see Transformation of tomato below).

Agrobacterium Preparation

Competent *Agrobacterium tumefaciens* strain LBA4404 cells with an OD_{600} of 0.1 were transformed with the construct described above using electroporation. A single colony from transformed *Agrobacterium* cells was inoculated into 10 mL LB broth with 50 mg l^{-1} kanamycin and grown for 24 h at 28 °C. An aliquot of the cultured cells was subsequently inoculated into 50-mL LB with 50 µg l^{-1} kanamycin and grown for another 24 h. This culture was harvested and used for the transformation described below.

Transformation of Tomato

The tomato transformation was modified from Wang and Campbell.[29] Tomato seeds (*Solanum lycopersicum*) cv Micro-Tom and Micro-Tina from Tomato Growers Supply Company, Fort Meyers, FL were surface sterilized in 40 mL of 25% bleach with 2 drops of tween 20 for 15 min and rinsed 5-7 times with distilled water before plating in seed germination medium (MS salt 4.3 g l^{-1}, Nitsch vitamin 1 mL l^{-1}, sucrose 30 g l^{-1} and agar 6 g l^{-1}, pH 5.8). Hypocotyls and cotyledon leaves of 7-10 days old seedlings were used for transformation. Three days before transformation, *Agrobacterium* culture was started in 20 mL LB medium supplemented with 50 mg l^{-1} kanamycin. A day before transformation, hypocotyl and cotyledon leaves were cut from seedlings at the petioles and the tip using a sterile razor on the co-cultivation media (MS salt 4.3 g l^{-1}, thiamine-HCL 0.4 mg l^{-1}, Myo-inositol 100 mg l^{-1}, sucrose 30 g l^{-1}, 2,4-D 0.2 mg l^{-1}, agar 6 g l^{-1} and kinetin 0.1 mg l^{-1}) with sterile filter paper laid on the surface. The bacteria from the LB broth were harvested by centrifugation and resuspended in co-cultivation media (without agar). Leaves from overnight incubation were scraped, mixed with bacteria, and incubated at room temperature for 30 min with occasional mixing. The bacterial suspension was then drained, and the leaves were dried on sterile paper

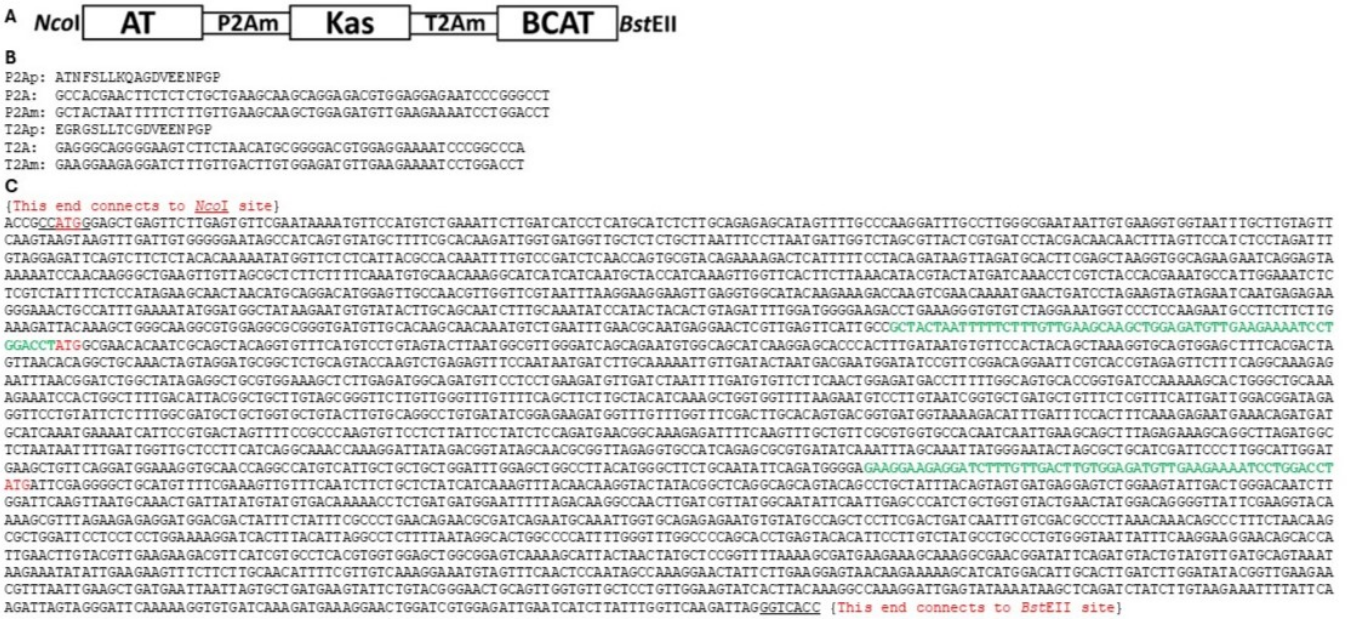

Figure 1. Tricistronic overexpression cassette. (A) Schematic of the tricistronic cassette. (B) P2Am and T2Am sequences used to facilitate cleavage of the three proteins. (C) DNA sequence of the cassette (P2Am and T2Am are in bold/green). BCAT:branched-chain amino acid aminotransferase; Kas:ketoacyl-ACP synthase; CS/AT/*PUN1:capsaicin* synthase/acyltransferase.

towels and returned to co-cultivation media with filter paper on the surface. These plates were sealed with micropore tape and incubated in the dark at room temperature for 3 days. After 3 days of co-cultivation, leaves were placed on regeneration medium (MS salts 4.3 g l^{-1}, Nitsch vitamin mL l^{-1}, sucrose 30 g l^{-1}, zeatin 1.5 mg l^{-1}, IAA 0.2 mg l^{-1}, carbenicillin 400 mg l^{-1}, hygromycin 30 mg l^{-1}, agar 6 g l^{-1} and pH 5.8) and incubated under natural day/light cycle for 3-8 weeks with media change every 3 weeks. Shoots will regenerate during this period. Regenerated shoots were transferred to rooting medium (MS salts 4.3 g l^{-1}, Nitsch vitamin 1mL l^{-1}, sucrose 30 g l^{-1}, IBA 0.5 mg l^{-1}, carbenicillin 400 mg l^{-1}, hygromycin 30 mg l-1, agar 6 g l^{-1} and pH 5.8) for further growth and rooting and then transferred to soil after acclimatization for few days.

Transformation Confirmation

Transformation and insertion of the transgene were confirmed by plant regeneration on hygromycin-containing regeneration media and then by PCR using hygromycin primers (forward: GATGTTGGCGACCTCGTATT and reverse: GATGTAGGAGGGCGTGGATA) on DNA from transgenic plants.

RNA Extraction

Total RNA was extracted using TRIzol®.[30] From T_0 transgenic plants, RNA was extracted for RT-PCR. Leaf samples from young transgenic plants were bulked to isolate RNA. Fresh 50-100 mg of leaf tissue was frozen in liquid N_2 and ground to powder using a mortar and pestle. One millilitre of TRIzol® reagent was used to homogenise 50-100 mg of tissue. After 5 min of incubation, 0.2 mL of chloroform was added to the sample and further incubated for 3 min. The mixture was centrifuged at 4 °C for 15 min. The supernatant was transferred to a new tube mixed with 0.5 mL of isopropanol and incubated for 10 min. After centrifuging at 4°C for 5 min, the supernatant was discarded, and the RNA pellet was mixed with 1 mL of 75% ethanol and centrifuged for 5 min at 4°C. The supernatant was discarded, and the pellet was dried by inverting tubes on clean filter paper for 10 min. Around 25-30 µL of DNase/RNase-free water was used to dissolve the RNA. An aliquot of this RNA were treated with DNase I at 37 °C for 10 min. This followed phenol:chloroform extraction with isopropanol precipitation and two 75% ethanol washes. The purity of the RNA was treated with DNase I and quantified using Nanodrop. Samples with 260/280 values between 1.9 and 2.1 were used for further analysis.

RT-PCR

Semiquantitative RT-PCR was performed to confirm transgene expression using qScript® XLT One-Step RT-PCR kit from QuantaBio in Applied Biosynthesis 2720 Thermocycler. Manufacturer's guidelines were followed for reaction setup and thermocycler procedures. In short, RT-PCR was performed for RNA of both transgenic and control plants in 10 µL volume. All reagents, RNA, and primers were thawed on ice for 15 min, and the reaction was set up on ice. For each 10 µL reaction, 5 µL one-step ToughMix (20X), 0.2 µL each of forward and reverse

primers, 0.4 µL of qScript® XLT One-Step reverse transcriptase (25X), 2.2 µL of nuclease free water and 2 µL of RNA were added and mixed. The PCR plate was then spun briefly to remove any bubbles and collect the contents at the bottom of the well. In the thermocycler, RT-PCR was programmed as follows: cDNA synthesis at 48°C for 20 min, initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 20 sec, annealing at 56°C for 30 sec, and extension at 72°C for 1 min. This was followed by 72°C for 5 min and 4°C for forever. The PCR product was run on 1% agarose gel along with loading dye to visualize the presence of the transgene in RNA.

Phenotypic Observations

Transgenic tomatoes were regularly checked for any visual phenotypic changes in their fruit shape and surface texture from early fruit stages to late maturity stages. Two people tasted the spiciness of ripened tomato fruits in the laboratory.

RESULTS AND DISCUSSION

Transformation of Tomato

Two tomato lines (Micro-Tina and Micro-Tom) were used in the transformation studies. The construct with the 35S promoter was used to transform Micro-Tina, and the construct with the E8 promoter was used to transform Micro-Tom. Although the number of infected leaves was not counted, regeneration of callus from leaves was variable for both Micro-Tina and Micro-Tom lines. Micro-Tom showed an early advantage with better germination and faster growth than Micro-Tina (data not collected). During callus regeneration, the adaxial side of cotyledon leaves increased the chance of regeneration. Once calli started to produce shoots, they were transferred to a Magenta box to have more room to grow. In total, 30 transgenic plants from the 35S vector and 35 transgenic lines from the E8 vector were generated.

Confirmation of Transgene Insertion

Genomic DNA was extracted from the leaves of transgenic and control tomato plants for PCR. Hygromycin primers were used to confirm the presence of the transgene in the T_0 generation of transgenic plants (Figure 2). Hygromycin bands were observed in both Micro-Tina and Micro-Tom transgenic lines but were absent from control plants, confirming the insertion of the transgene into the tomato genome.

Gene Expression

Expression of the transgene was confirmed by semiquantitative RT-PCR using pepper gene-specific primers. For each primer set, once the primers were designed, they were BLASTed against the tomato sequence to confirm their absence from

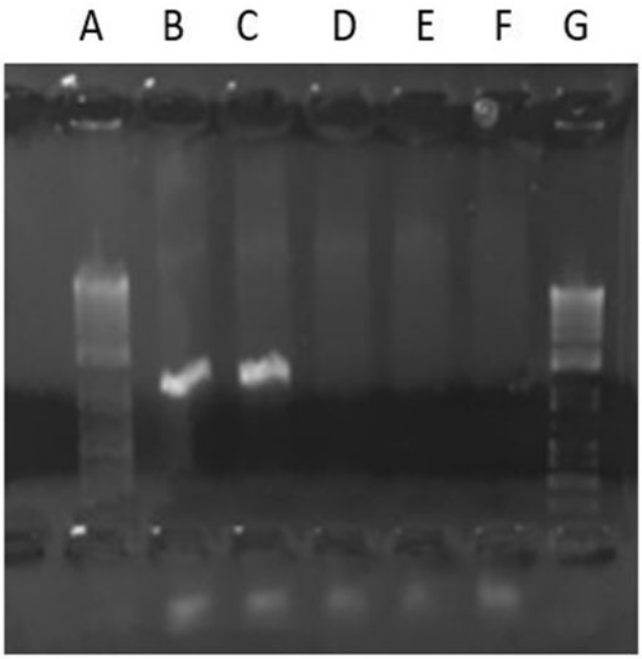

Figure 2. PCR confirmation of transgene. Lanes A and G-1 kb ladder; B, transgenic line 1; C, transgenic line 2; D, Micro-Tina control; E, Micro-Tom control and F-PCR negative control.

tomato. For each of the three genes, one specific primer set was designed. RT-PCR was performed using these primers on RNA derived from control and transgenic plants. Gel electrophoresis of the RT-PCR product showed gene bands for all three target genes (*AT, BCAT*, and *Kas*) in both transgenic lines tested, whereas the control plant did not have any transgene expression (Figure 3). This shows that all three pepper genes were integrated with the tomato genome and were being expressed.

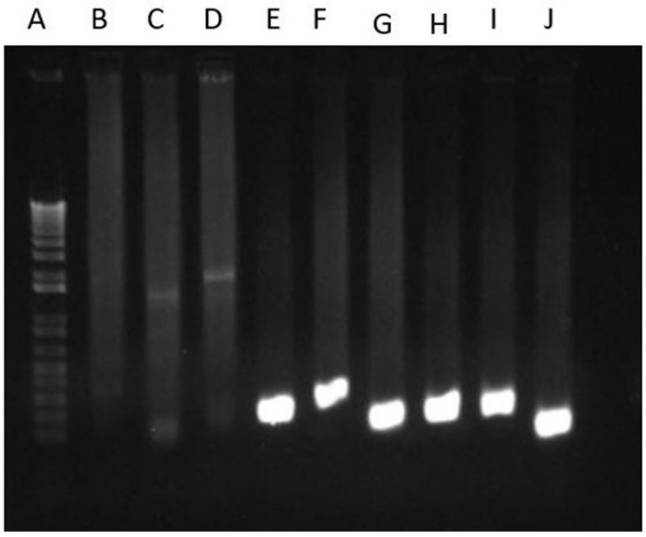

Figure 3. Gene expression in transgenic plants. Lanes A: ladder; B, C, D: control for *AT, BCAT*, and *Kas* genes; E, F, G: transgenic line 1 for *AT, BCAT*, and *Kas*; H, I, J: transgenic line 2 for *AT, BCAT*, and *Kas*.

Phenotypic Changes

Transgenic plants were observed for any phenotypic structural changes in plants and fruits. There were no overwhelming changes that were consistent throughout all transgenic lines. However, some transgenic tomato plants did produce fruits with pepper-like rough skin (Figure 4).

Figure 4. Some transgenic tomato fruits showed rough skins at the T_0 generation before turning red.

Spiciness

Ripened transgenic tomato fruits were tasted for a hint of heat. Two people independently tasted the juice from the same fruit to determine whether it was hot. There was consensus that none of the transgenic tomato fruits carried any spiciness. Although our results from RT-PCR suggested the expression of all three genes in transgenic plants, the lack of heat in fruit suggests either that there is no detectable increase in capsaicin production or that the biosynthetic pathway requires more than three genes to be overexpressed.

Tomato as a biofactor for the production of secondary metabolites is not a new concept. Butelli et al.[31] could use tomato to produce betalain (food colour). Other studies have used tomatoes to increase the production of various flavonoids[32] and phenylpropanoid compounds like resveratrol and genistin[33]. Tomato fruits are rich in primary metabolites such as citrate, malate, and ascorbic acids and secondary metabolites such as carotenoids, phenylpropanoid and terpenoids.[34] This suggests the presence of a basic biosynthetic pathway for each in tomatoes, which has led many to believe that tomatoes can be used as a chassis to produce various economically as well as pharmacologically important metabolites such as retinol (Vitamin A) through B-carotene biosynthesis and dioscin through steroidal glycoalkaloid production.[35,36]

The presence of the defunct capsaicinoid biosynthesis pathway in tomatoes offers an opportunity to produce capsaicin in this plant. This will alleviate the problems associated with pepper farming, such as varying levels of pungency, environmental distress, slow seed germination and long life cycle, as well as high levels of soil-borne diseases and nematode infection.[2] Both pepper and tomato, being members of the same nightshade family (Solanaceae), have major conserved synteny with a basic chromosome number of x=12 in both species and share many traits between them.[17] Comparative genomics revealed the presence of all necessary capsaicinoid genes in tomato with varying expression.[3] Overexpression of those less expressed genes in tomatoes should, in theory, make tomato fruit spicy, but other regulatory factors cannot be excluded.

By inserting three capsaicinoid genes (*BCAT, Kas* and *AT*), we aimed to activate the capsaicinoid pathway in tomato. The *Agrobacterium*-mediated transformation method was successful in the delivery of all three genes, as evidenced by the regeneration of hygromycin-resistant transgenic plants and the expression of three pepper genes, as seen in RT-PCR. This, however, did not result in any increase in capsaicin synthesis or accumulation in tomato fruits. The generation of transgenic tomato plants expressing pepper gene, however, is a significant step towards the generation of spicy tomato. There could be many reasons why our plants did not produce spicy fruits. It could be that there is too little capsaicin to be detected by tasting, or the three genes might not be enough to activate the whole capsaicin biosynthesis pathway as it comprises more than 51 gene families.[17] Gene duplication during capsicum evolution meant that the biosynthesis pathway has up to 13 pepper-specific duplication compared to tomato[17], also tissue specific and developmental expression of genes involved in capsaicinoid biosynthesis[3] could play a role in making tomato spicy.

To the best of our knowledge, the only species other than peppers metabolically engineered to produce capsaicin, the main capsaicinoid in peppers[37], is the baker's yeast *Saccharomyces cerevisiae*. Zhao et al. [38] overexpressed in yeast all the genes for the enzymes catalysing seven steps that produce vanillylamine from phenylalanine[17], *Kas*, *thioesterase* (*Fat*), and *ACL* to reconstruct the fatty acid pathway, as well as *CS/AT*. Culturing the engineered yeast for 120 h in a 1 L fermenter containing 20 g/L glucose, they were able to produce 80.23 µg/L capsaicin in *S. cerevisiae*.[38]

CONCLUSION

To conclude, in this study, we showed that P2Am and T2Am sequences can be used in the overexpression of multiple genes in tomatoes. Future work in tomatoes may include metabolic profiling to examine the changes in metabolites in transgenic plants.

Peer Review: Externally peer-reviewed.

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REFERENCES

1. Aza-González C, Nunez-Palenius HG and Ochoa-Alejo N. Molecular biology of capsaicinoid biosynthesis in chili pepper (*Capsicum* spp.). *Plant Cell Rep*. 2011;30:695–706.
2. Naves ER, Silva L De A, Sulpice R, et al. Capsaicinoids: Pungency beyond *Capsicum. Trends Plant Sci*. 2019;24:109-120.
3. Kim S, Park M, Yeom SI, et al. Genome sequence of the hot pepper provides insights into the evolution of pungency in *Capsicum* species. *Nat Genet.* 2014;46:270-278.
4. Simonne AH, Simonne EH, Eitenmiller RR, Mill HA, Green NR. Ascorbic acid and pro-vitamin A contents in unusually colored bell peppers (*Capsicum annuum* L.). *J Food Comp Anal*. 1997;10:299-311.
5. Kothari SL, Joshi A, Kachhwaha S, Ochoa-Alejo N. Chilli pepper- A review on tissue culture and transgenesis. *Biotech Adv*. 2010;28:35-48.
6. Ravishankar GA, Suresh B, Giridhar P, Rao SR, Johnson TS. Biotechnological studies on *Capsicum* metabolite production and plant improvements. In: De AK (ed.), *Capsicum*: the genus *Capsicum*. London: CRC Press. 2003.
7. Hardy G. Nutraceuticals and functional foods: Introduction and meaning. *Nutrition* 2000;16:688-689.
8. Lee SG, Kim SK, Lee HJ, Lee HS, Lee JH. Impact of moderate and extreme climate change scenarios of growth, morphological features, photosynthesis, and fruit production of hot pepper. *Ecol Evol*. 2018;8:197-206.
9. Demir I and Ellis RH. Development of pepper (*Capsicum annuum*) seed quality. *Ann Appl Biol*. 1992;121:385-399.
10. Zewdie Y and Bosland PW. Evaluation of genotype, environment, and the genotype-by-environment interaction for capsaicinoids in *Capsicum annuum L. Euphytica*. 2000;111:185-190.
11. Onus AN, and Pickersgill B. Unilateral incompatibility in *Capsicum* (Solanaceae): Occurrence and taxonomic distribution. *Ann Bot*. 2004;94:289-295.
12. Heidmann I and Boutilier K. Pepper, sweet (*Capsicum annuum*). *Methods Mol Biol.* 2015;1223:321-334.
13. Min J, Shin SH, Jeon EM, Park JM, Hyun JY and Harn CH. Pepper, chilli (*Capsicum annuum*). *Method Mol Biol*. 2015;1223:311-320.
14. Li Y, Wang H, Zhang Y and Martin C. Can the world's favorite fruit, tomato, provide an effective biosynthetic chassis for high-value metabolites? *Plant Cell Rep.* 2018;37:1443-1450.
15. Peixoto JV, Neto CM, Campos LF, Dourado WS, Nogueira AP, Nascimento AD. Industrial tomato lines: Morphological properties and productivity. *Genet Mol Res.* 2017;16:1-15.
16. Sarkinen T. Bohs L, Olmstead RG and Knapp S. A phylogenetic framework for evolutionary study of the nightshades (Solanaceae): A dated 1000-tip tree. *BMC Evol Biol*. 2013;13:214. doi:10.1186/1471-2148-13-214
17. Qin C, Yu C, Shen Y, et al. Whole-genome sequencing of cultivated and wild peppers provides insights into *Capsicum* domestication and specialization. *Proc Natl Acad Sci USA.* 2014;111:5135-5140.
18. Food and Agriculture Organization of the United Nations. FAO Statistical Yearbook 2015: World Food and Agriculture, FAO.
19. Jenkins T. Bovi A and Edwards R. Plants: Biofactories for a sustainable future? *Philos Trans A Math Phys Eng Sci*. 2011;369:1826-1839.
20. Stewart C, Kang BC, Liu K, et al. Pun1 gene for pungency in pepper encodes a putative acyltransferase. *Plant J*. 2005;42:675-688.
21. Kim M, Kim S, Kim S and Kim BD. Isolation of cDNA clones differentially accumulated in the placenta of pungent pepper by suppression subtractive hybridization. *Mol Cells.* 2001;11:213-219.
22. Tanaka Y, Nakashima F, Kirii E, Goto T, Yoshida Y, Yasuba KI. Difference in capsaicinoid biosynthesis gene expression in the pericarp reveals elevation of capsaicinoid contents in chilli peppers *(Capsicum chinense). Plant Cell Rep.* 2017;36:267-279.
23. Sanjana NE, Cong L, Zhou Y, Cunniff M, Feng G and Zhang F. A transcriptional activator-like effector toolbox for genome engineering. *Nat Protoc.* 2012;7:171-192.
24. Songstad DD, Petolino JF, Voytas DF and Reichert NA. Genome editing in plants. *Crit Rev Plant Sci.* 2017;36:1-23.
25. Schouten HJ and Jacobsen E. Cisgenesis and intragenesis, sisters in innovative plant breeding. *Trends Plant Sci.* 2008;13:260-261.
26. Hou H, Atlihan N and Lu ZX. New biotechnology enhances the application of cisgenesis in plant breeding. *Front Plant Sci.* 2014;5:389. doi:10.3389/fpls.2014.00389
27. Osborn MJ, Panoskaltsis-Mortari A, McElmurry RT, et al. A picornaviral 2A-like sequence-based tricistronic vector allowing for high-level therapeutic gene expression coupled to a dual-reporter system. *Molecular Ther.* 2008;12:569-574.
28. Grote A, Hiller K, Scheer M, et al. JCat: A novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Res.* 2005;33:W526-531.
29. Wang Y-H, Campbell MA. *Agrobacterium*-mediated transformation of tomato elicits unexpected flower phenotypes with similar gene expression profiles. *PLoS ONE* 2008;3: e2974. doi:10.1371/journal.pone.0002974
30. Bleeker PM, Spyropoulou EA, Diergaarde PJ, et al. RNA-seq discovery, functional characterization, and comparison of sesquiterpene synthases from *Solanum lycopersicum* and *Solanum habrochaites trichomes. Plant Mol Biol*. 2011;77:323-336.
31. Butelli E, Titta L. Giorgio M, et al. Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nat Biotech*. 2008;26:1301-1308.
32. Tzin V, Rogachev I, Meir S, et al. Tomato fruits expressing bacterial feedback insensitive 3-deoxy-d-arabino-heptuloconate 7-phosphate synthase of shikimate pathway possess enhanced levels of multiple specialized metabolites and upgraded aroma. *J Exp Bot.* 2013;64: 4441-4452.

33. Zhang Y, Butelli E, Alseekh S, et al. Multi-level engineering facilitates the production of phenylpropanoid compounds in tomato. *Nat Commun*. 2015;6:8635-8655.
34. Siddiqui MW, Ayalazavala JF, Dhua RS. Genotypic variation in tomatoes affecting processing and antioxidant attributes. *Crit Rev Food Scie Nutr.* 2015;55:1819-1835.
35. Ru M, Wang K, Bai Z, et al. Molecular cloning and characterization of two enzymes involved in the rosmarinic acid biosynthesis pathway of *Prunella vulgaris* L. *Plant Cell Tissue Organ Cult.* 2016;128:381-390.
36. Sonawane PD, Pollier J, Panda S, et al. Plant cholesterol biosynthetic pathways overlaps with phytosterol metabolism. *Nat Plants*. 2016;3:16205-16218.
37. Davis CB, Markey CE, Busch MA, Busch KW. Determination of capsaicinoids in habanero peppers by chemometric analysis of UV spectral data. *J Agric Food Chem.* 2007;55:5925-5933.
38. Zhao J, Li Y, Yi L, Zhang G, Ye B. Yeast-based whole-cell factory for the ecofriendly synthesis of vanillylamine as a critical step toward capsaicin production. *ACS Sustain. Chem Eng.* 2023;11:7683-7691.

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