Bioengineering of the plant culture of *Capsicum frutescens* with vanillin synthase gene for the production of vanillin

Marcus Jenn Yang Chee[1], Grantley W. Lycett[2], Teng-Jin Khoo[3], Chiew Foan Chin[1*]

1. School of Biosciences, Faculty of Science, University of Nottingham, Malaysia Campus, 43500 Semenyih, Selangor Darul Ehsan, Malaysia
2. School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, United Kingdom
3. School of Pharmacy, Faculty of Science, University of Nottingham, Malaysia Campus, 43500 Semenyih, Selangor Darul Ehsan, Malaysia

chiew-foan.chin@nottingham.edu.my

[*]Corresponding author

Dr. Chiew Foan Chin

Mailing address: School of Biosciences, Faculty of Science, University of Nottingham, Malaysia Campus, 43500 Semenyih, Selangor Darul Ehsan, Malaysia

Phone: +60-3-8924 8216

E-mail: chiew-foan.chin@nottingham.edu.my

Abstract

Production of vanillin by bioengineering has gained popularity due to consumer demand towards vanillin produced by biological systems. Natural vanillin from vanilla beans is very expensive to produce compared to its synthetic counterpart. Current bioengineering works mainly involve microbial biotechnology. Therefore, alternative means to the current approaches are constantly being explored. This work describes the use of vanillin synthase (*Vp*VAN), to bioconvert ferulic acid to vanillin in a plant system. The *Vp*VAN enzyme had been shown to directly convert ferulic acid and its glucoside into vanillin and its glucoside, respectively. As the ferulic acid precursor and vanillin were found to be the intermediates in the phenylpropanoid biosynthetic pathway of *Capsicum* species, this work serves as a proof-of-concept for vanillin production using *Capsicum frutescens* (*C. frutescens* or hot chili pepper). The cells of *C. frutescens* were genetically transformed with a codon optimized *VpVAN* gene via biolistics. Transformed explants were selected and regenerated into callus. Successful integration of the gene cassette into the plant genome was confirmed by polymerase chain reaction. High performance liquid chromatography was used to quantify the phenolic compounds detected in the callus tissues. The vanillin content of transformed calli was 0.057% compared to 0.0003% in untransformed calli.

Keywords: vanillin; ferulic acid; vanillin synthase; *Vp*VAN; *Capsicum frutescens*

1. Introduction

Vanilla is one of the most important flavors in the food and beverage industries, and it is also used in perfumery and pharmaceutical products. Natural vanilla extract from *Vanilla planifolia* (*V. planifolia*) or *Vanilla tahitensis* is made up of over 200 components, the major compound being vanillin (4-hydroxy-3-methoxybenzaldehyde). Other compounds include vanillic acid, p-hydroxybenzaldehyde, p-hydroxybenzoic acid, sugars and lipids [1]. Medically, it was reported that vanillin is able to suppress the proliferation of cancer cells and prevent chemically and physically induced mutagenesis [2]. It was also reported that vanillin exhibits antimicrobial properties [3].

Despite the usefulness of vanilla or more specifically, vanillin, natural vanillin is very expensive to produce. This is largely attributed to the laborious and time-consuming process to extract vanillin from vanilla beans. In the market, only a small portion of vanilla flavoring is derived from natural vanilla beans due to the low supply of vanilla beans, which is often subjected to extensive price fluctuations. The market price of natural vanilla has recently soared from over USD 200 per kg to USD 400–500 per kg in 2016, which is more than a ten times increase from its lowest price at just USD 20 per kg ten years ago [4]. Only 2000 tons of the global demand, which is more than 15,000 tons, is provided by vanilla beans [5]. The rest is supplied by synthetic vanillin produced from lignin and eugenol. Nevertheless, the market share of natural vanilla is believed to be not affected by their artificial counterparts due to the shift in demand towards food regarded as natural and organic. US and EU labeling regulations allow only goods produced using natural vanilla to be labeled “vanilla” [6,7]. In addition, bioengineered vanillin from plant tissues and microorganisms is still of low success because of the high cost incurred in culture fermentation and the requirement to optimize various culture conditions [8,9].

Recently, the vanillin biosynthetic pathway in *V. planifolia* has been explored by Gallage and co-workers [10]. An enzyme named vanillin synthase (*Vp*VAN) was found to directly convert ferulic acid and its glucoside to vanillin and its glucoside, respectively, based on transient expression in tobacco and stable expression in barley. *Vp*VAN is a type of hydratase or lyase that shows high sequence similarity to that of cysteine proteinases. Besides its discovery in *V. planifolia*, vanillin synthase-like enzymes in bacteria had been reported by Pometto and Crawford [11], and by Narbad

and Garsson [12]. Similar enzymes in fungi have also been reported by Hansen and co-workers [13]. The enzymes were generally referred to as aldehyde oxidase, CoA ligase, dehydrogenase, hydratase or reductase.

This research explores a plant-based alternative to the current vanillin production systems by the heterologous expression of a *VpVAN* gene in callus cultures of *Capsicum frutescens* (*C. frutescens*) L. var. Hot Lava (chili). Ferulic acid (4-hydroxy-3-methoxycinnamic acid) and vanillin were found to be the precursors for the biosynthesis of capsaicin in chili (Fig. 1) [14]. Thus, the constitutive expression of *VpVAN* could potentially enable bioconversion of endogenous ferulic acid to vanillin in the callus cultures of *C. frutescens* at a higher level compared to the untransformed callus. This would potentially lead to the production of natural, pure vanillin using an alternative bioengineered plant-based system in another food crop.

2. Materials and Methods

Construction of expression vector

The vanillin synthase gene from *Vanilla planifolia* (*VpVAN*) was codon optimized according to codon usage of *Nicotiana benthamiana* based on the DNA coding sequence provided by Gallage and co-workers [10]. The gene was then commercially synthesized (Integrated DNA Technologies, Singapore) into a holding vector pIDT-AMP:35Sp-*VpVAN*-V5. Upstream of the *VpVAN* gene was the cauliflower mosaic virus 35S promoter (35Sp), flanked by a *Pst*I restriction site at the 5′ end, while downstream of the gene was a V5 epitope, flanked by a *Bam*HI restriction site at the 3′ end. The holding vector and the target destination vector, pcDNA6.2/V5-pL-DEST, were separately cleaved with *Pst*I and *Bam*HI restriction endonucleases. The linearized pcDNA vector backbone and the 35Sp-*VpVAN*-V5 gene cassette were ligated using T4 DNA ligase to produce pcDNA6.2::(35Sp-*VpVAN*-V5)Δ*ccd*B (Fig. 2). To check on the efficacy of transgene expression in the destination vector, a separate gene cassette containing the gene of a synthetic green fluorescence protein (sGFP) was cloned into the destination vector in place of the *VpVAN*-V5 gene cassette, but under the regulation of the same 35Sp promoter. The resulting pcDNA6.2::(35Sp-*sGFP*)Δ*ccd*B was delivered into explants and the expression of sGFP was observed using a stereo microscope (Nikon SMZ1000) with a blue light fluorescent attachment.

Bacterial strain and growth media

The expression vector was propagated using One Shot *ccd*B Survival 2 T1^{R} chemically competent *Escherichia coli* (Invitrogen) following transformation by heat shock. The bacteria were cultured on Luria Bertani (LB) agar, then in LB broth containing 100 µg/mL ampicillin and 15 µg/mL chloramphenicol as the selective antibiotics. Purification of the expression vector was carried out using Hybrid-Q Plasmid Rapidprep kit (GeneAll) according to manufacturer's protocol.

Plant material

Seeds of *Capsicum frutescens* L. cv. Hot Lava were surface sterilized using 70% (v/v) ethanol, followed by washing in sterile distilled water. They were then soaked in 20% (v/v) commercial Clorox (1.05% sodium hypochlorite), and were subsequently washed twice in sterile distilled water. The sterilized seeds were germinated on Murashige and Skoog (MS) agar (4.42 g/L MS basal salt, 20 g/L sucrose, and 3.5 g/L agar (Phytagel)) for two weeks. Hypocotyls of germinated seedlings were excised and incubated in the dark overnight prior to particle bombardment.

Particle bombardment

The expression vector was coated onto 1.6 µm gold particles by mixing of the DNA and the gold particles with spermidine and calcium chloride with constant vortex. Coated gold particles were pelleted and the resulting supernatant was removed, followed by washing with 70% (v/v) ethanol. Subsequently, the particles were washed with 100% (v/v) ethanol and were resuspended in 100% (v/v) ethanol prior to loading on macrocarriers. Particle bombardment was performed on the explants using a PDS-1000/He system at 1350 psi helium pressure in a vacuum chamber at 28 mm mercury pressure. Three replicates of twenty explants per replicate were subjected to the bombardment. The target distance was set at 6 cm. Bombarded explants were then incubated in the dark overnight for recovery.

Selection and regeneration of putative transformants

After an overnight incubation, the bombarded explants were transferred to selective medium containing 4.42 g/L MS basal salt, 30 g/L sucrose, 3.5 g/L agar (Phytagel), 0.1 mg/L 1-naphthaleneacetic acid (NAA), 5.0 mg/L 6-benzylaminopurine (BAP) and 0.2 mg/L blasticidin S. The explants were incubated at 25°C in 16 hours light and 8 hours dark photoperiod for one month. Putative transformants that survived and

proliferated were then transferred to fresh selective medium and were incubated for another month.

Isolation of plant genomic DNA

Calli of *Capsicum frutescens* transformants were ground into fine powder in liquid nitrogen and extracted in 200 mM Tris-Cl (pH 7.5), 250 mM sodium chloride, 25 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% (w/v) sodium dodecyl sulfate (SDS). Purification was done with Tris-saturated phenol, followed by the centrifugation at 13,000 rpm and the addition of an equal volume of chloroform–isoamyl alcohol (24:1, v/v) to the aqueous phase. The mixture was centrifuged. The DNA in the aqueous phase was precipitated in 99% (v/v) isopropanol and subsequently, the isopropanol was removed and the DNA pellet was washed with 70% (v/v) ethanol. Finally, the DNA was resuspended in sterile nuclease free water.

Confirmation of transformants by polymerase chain reaction (PCR)

Amplification of *VpVAN* by PCR from the extracted genomic DNA of calli was performed using primers 5′-AGG ACG TCT CGT ACA CCA TGG ATG GCA GCT AAG CTC CTC TTC-3′ and 5′-GGT CAA AAT GAG ACG GGG ATC CGC TAG TGA TGG TGG TGG TGA TGC ACA GCC ACA ATG GGA TAA GAT GC-3′ (0.3 µM each). The same primers were also used to verify the presence of *VpVAN* gene in genomic DNA from leaves of *V. planifolia*. PCR reactions were carried out using recombinant Taq polymerase (Invitrogen) and Taq polymerase buffer with 1.5 mM magnesium chloride, 0.3 mM deoxynucleotide mix (dNTPs). Thermal cycling conditions were: initial denaturation at 95°C for 3 min; 25 times of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and elongation at 72°C for 1.5 min; and final elongation at 72°C for 5 min. Amplified DNA was separated by gel electrophoresis on 1% (w/v) agarose gel in 1X Tris-acetate-EDTA (TAE) buffer and stained using SYBR Safe DNA gel stain (Invitrogen).

Extraction of Phenolic Compounds

Phenolic compounds from callus cultures were extracted using maceration and sonication. Two grams of callus tissue was weighed and ground with 80% (v/v) ethanol, followed by sonication. Suspension with ethanol and sonication was repeated twice. All liquid extracts were collected. Extraction solvent was removed by rotary evaporation. Target compounds were then redissolved in 80% (v/v) methanol.

High Performance Liquid Chromatography (HPLC)

All callus extracts were filtered through a 0.45 µm syringe filter prior to injection into the HPLC system. A gradient HPLC was performed with a mobile phase ratio changing from 1:3 to 1:1 methanol–1% acetic acid over 15 min. Flow rate was set at 1 mL/min. The HPLC column used was Hypersil GOLD (Thermo) C18 analytical column (250 × 4.6 mm ID, 5 µm particle size). Target phenolic compounds were detected using ultraviolet (UV) photodiode array at 260 – 325 nm wavelengths.

3. Results and discussion

Recombinant expression vector

To examine whether the ligation product (expression vector) pcDNA6.2::(35Sp-*VpVAN*-V5)Δ*ccd*B was successfully transformed into chemically competent *Escherichia coli*, polymerase chain reaction (PCR) was performed on the bacterial colonies grown overnight on Luria Bertani agar containing ampicillin and chloramphenicol. Colonies that showed the amplification of the 1133 bp *VpVAN* gene (in Fig. S1) were selected for subculture to propagate the expression vector. Subsequently, the expression vector that was extracted was subjected to another PCR of the *VpVAN* gene and to cleavage by *Pst*I and *Bam*HI restriction endonucleases for further verification (in Fig. S2). The double restriction digest gave the expected DNA bands of 6028 bp and 1460 bp. Additionally, the expression vector was sequenced and the resulting reads showed up to 100% sequence identity to that of the known cDNA sequence of *VpVAN* gene (data not shown).

Selection, regeneration and screening of plant transformants

The particle bombardment procedure with pcDNA6.2::(35Sp-*VpVAN*-V5)Δ*ccd*B achieved 5% transformation efficiency, whereby one out of twenty explants in each of the three replicates survived and proliferated into callus on the blasticidin selective media over two months (Fig. 3A). Explants that did not survive showed signs of shrinking and extensive browning with very little or no callus proliferation at all (Fig. 3B). Separately, transformation with pcDNA6.2::(35Sp-*sGFP*)Δ*ccd*B achieved 20% transformation efficiency and the surviving explants (Fig. 3C) showed the expression of sGFP (Fig. 3D). This demonstrated effective expression of the gene cassette transferred with the expression vector. In addition, the *sGFP* gene was a modified

GFP gene with a chromophore mutation at position 65, where serine was replaced with threonine, to give 100-fold higher fluorescence signal compared to the original jellyfish GFP [15].

Integration of the gene cassette into the plant genome was verified by PCR of *VpVAN* after the extraction of genomic DNA from the callus tissues. The gene was detected in the genomic DNA of all callus samples, hence indicating successful integration of the gene cassette into the plant genome (Fig. 4). PCR amplification of *VpVAN* was not achieved from the genomic DNA of non-transformed *C. frutescens* callus (Fig. 5). On the other hand, genomic DNA from the leaf of *V. planifolia* showed the amplification of *VpVAN*, indicating the presence of native vanillin synthase in the vanilla plant (Fig. 5). However, the endogenous *VpVAN* gene in *V. planifolia* is larger in size (~2500 base pairs) compared to the cDNA sequence provided by Gallage and co-workers [10] (1071 base pairs). This suggests the possible presence of introns that contribute additional base pairs to the genomic sequence. Preliminary sequencing of the *VpVAN* PCR product from the genomic DNA of *V. planifolia* and comparison of the sequence obtained with the known cDNA sequence of *VpVAN* revealed at least an intron of 909 base pairs in length (data not shown).

Levels of phenolic compounds in *C. frutescens* calli

Four target phenolic compounds—vanillin, vanillic acid, vanillin-β-D-glucoside, and ferulic acid—were analyzed using high performance liquid chromatography (HPLC). Maximum UV absorbance for the four compounds was measured at 280 nm, 260 nm, 270 nm, and 280 nm wavelengths, respectively. The retention times in chromatograms acquired for the target compounds were compared to those of the external standards (Fig. 6). Transformed calli produced vanillin at an average of 573.39 (±120.70) µg per gram tissue. This was equivalent to 0.057% of vanillin in the fresh callus. The amount of vanillin produced was significantly higher than that from the untransformed calli, which produced detectable levels of vanillin at an average of only 3.32 (±0.83) µg per gram tissue (0.0003%) (Fig. 7). The increase in vanillin level was 190 times. The *Vp*VAN enzyme could have catalyzed the bioconversion of vanillin from endogenous ferulic acid as free and bound ferulic acid is one of the most abundant phenylpropanoids in plant tissues. Being an important molecule in the plant cell wall, ferulic acid is present either as free homodimers or as dehydrodimers and dehydrotrimers esterified with proteins or sugars [16-18]. In a study by Yahiaoui and

co-workers [19], it was shown that the downregulation of cinnamyl alcohol dehydrogenase (CAD, an enzyme in lignin biosynthesis) in transgenic tobacco (*Nicotiana tabacum*) saw up to ten-fold increase in vanillin content (>4 µmol per gram of extracted xylem residue) compared to untransformed control. Looking at the capsaicinoid biosynthetic pathway, the suppression of a putative aminotransferase gene (*pAMT*) in *Capsicum frutescens* resulted in no vanillylamine production and very low levels of capsaicinoid, which is downstream of vanillin in the capsaicinoid biosynthetic pathway. This in turn stimulated an increase in metabolites, such as vanillin and vanillic acid [20]. Treatment of cell cultures of *Capsicum chinense* with 200 µM salicylic acid was shown to increase the activity of phenylalanine ammonia lyase (PAL, a key enzyme producing cinnamic acid in the phenylpropanoid pathway) and subsequently increased vanillin production almost 3 times higher than untreated control [21-22].

In our studies, ferulic acid was found at low levels in transformed (1.12 ±0.32 µg per gram tissue) and untransformed (1.53 ±0.01 µg per gram tissue) calli. It is possible that most of the ferulic acid in plant tissues was esterified in cell walls rather than being free dimers [17-18]. As such, it would not have been released during the extraction process. Other than that, free ferulic acid that appeared as intermediate in the phenylpropanoid pathway could have been instantly degraded or converted into other phenolic derivatives, such as feruloyl-CoA, vanillyl-CoA, 4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl CoA, vanillic acid and vanillin, based on the possible routes for bioconversion of ferulic acid to vanillin [16].

In spite of the low levels of ferulic acid detected, it is shown that the level of ferulic acid in transformed and untransformed tissues appeared to be similar, suggesting that there could be compensation of intracellular ferulic acid that was converted into vanillin. It is known that ferulic acid is a source of feruloyl-CoA in lignin biosynthesis. In a study of transgenic poplar (family Salicaceae), the downregulation of cinnamoyl CoA reductase (CCR, one of the key enzymes involved in the conversion of feruloyl-CoA from the general phenylpropanoid pathway to monolignols in lignin biosynthesis) resulted in a decreased flux of feruloyl-CoA to lignin, which in turn stimulated an increased flux of ferulic acid deposition [23-24]. In the case of vanillin biosynthesis, the ability of *Vp*VAN to release ferulic acid from plant cell wall material is still unknown. Hence, the biosynthesis of vanillin by *Vp*VAN could have taken place via ferulic acid and feruloyl-CoA in the

phenylpropanoid pathway. The diversion of ferulic acid for the synthesis of vanillin could have caused a reduced flux to lignin, thereby inducing a compensation of ferulic acid in return.

*Vp*VAN is able to catalyze the synthesis of vanillin and its glucoside from ferulic acid and its glucoside, respectively, as described by Gallage and co-workers [10]. Therefore, the detection of significant level of vanillin-β-D-glucoside (110.32 ±11.56 µg per gram tissue or 0.01%) as compared to the negative controls (6.63 ±7.92 µg per gram tissue or 0.0006%) suggests the presence of ferulic acid glucosides in the callus tissues that were used in the conversion by *Vp*VAN. This could be further confirmed in future studies. The detectable amount of vanillic acid (89.29 ±36.31 µg per gram tissue or 0.009%) in transformed calli also suggests the presence of intermediary forms of vanillic acid glucosides and vanillic acid in the vanillin biosynthetic pathway in the plant tissues.

4. Conclusion

The efficacy of the gene cassette harboring the *VpVAN* gene and the expression vector was demonstrated by the constitutive expression of green fluorescence protein. Plant transformants were selected effectively by the blasticidin antibiotics used in the selective media supplemented with NAA and BAP for plant regeneration. The heterologous expression of the *VpVAN* gene in *C. frutescens* had resulted in almost 200 times increase in the levels of vanillin and vanillin glucoside in transformed callus tissues compared to untransformed tissues. On the other hand, the endogenous level of ferulic acid in transformed tissues was low, which was similar to that of the untransformed tissues as negative controls.

5. Acknowledgments

This work was funded by the Malaysian Ministry of Science, Technology and Innovation through the eScience Fund (02-02-12-SF0130), and by the Malaysian Ministry of Education through the MyBrain15 PhD scholarship programme.

6. Literature Cited

1. Rao, S. R. and Ravishankar, G. A. (2000) Vanilla flavor: production by conventional and biotechnological routes. J. Sci. Food Agric. 80, 289–304.
2. Cheng, W-Y., Hsiang, C-Y., Bau, D-T., Chen, J-C., Shen, W-S., Li, C-C., Lo, H-Y., Wu, S-L., Chiang, S-Y. and Ho, T-Y. (2007) Microarray analysis of vanillin-regulated gene expression profile in human hepatocarcinoma cells. Pharmacol. Res. 56, 474–482.
3. Cerrutti, P., Alzamora, S. M. and Vidales, S. L. (1997) Vanillin as an antimicrobial for producing shelf-stable strawberry puree. J. Food Sci. 62, 608–610.
4. Market Insider, ITC (2016) Frightening ride forecast for vanilla. International Trade Centre. Available at http://www.intracen.org/itc/blogs/market-insider/Frightening-ride-forecast-for-vanilla/. Accessed September 19, 2016.
5. Rana, R., Mathur, A., Jain, C. K., Sharma, S. K. and Mathur, G. (2013) Microbial production of vanillin. Int. J. Biotechnol. Bioeng. Res. 4, 227–234.
6. de Melo, J. (2015) Developing Countries in the World Economy. World Scientific, Singapore.
7. Sabisch, M. and Smith, D. (2014) The complex regulatory landscape for natural flavor ingredients. Sigma-Aldrich. Available at http://www.sigmaaldrich.com/technical-documents/articles/white-papers/flavors-and-fragrances/natural-flavor-ingredients-regulations.html#definitions. Accessed December 1, 2015.
8. Converti, A., Aliakbarian, B., Domínguez, J. M., Vazquez, G. B. and Perego, P. (2010) Microbial production of biovanillin. Braz. J. Microbiol. 41, 519–530.
9. Longo, M. A. and Sanromán, M. A. (2006) Production of food aroma compounds: microbial and enzymatic methodologies. Food Technol. Biotechnol. 44, 335–353.
10. Gallage, N. J., Hansen, E. H., Kannangara, R., Olsen, C. E., Motawia, M. S., Jørgensen, K., Holme, I., Hebelstrup, K., Grisoni, M. and Møller, B. L. (2014) Vanillin formation from ferulic acid in *Vanilla planifolia* is catalysed by a single enzyme. Nature Comm. 5, 4037 doi: 10.1038/ncomms5037.
11. Pometto, A. L. III and Crawford, D. L. (1983) Whole-cell bioconversion of vanillin to vanillic acid by *Streptomyces viridosporus*. Appl. Environ. Microbiol. 45, 1582–1585.

12. Narbad, A. and Gasson, M. J. (1998) Metabolism of ferulic acid via vanillin using a novel CoA-dependent pathway in a newly-isolated strain of *Pseudomonas fluorescens*. Microbiology 144, 1397–1405.
13. Hansen, E. H., Møller, B. L., Kock, G. R., Bünner, C. M., Kristensen, C., Jensen, O. R., Okkels, F. T., Olsen, C. E., Motawia, M. S. and Hansen, J. (2009) De novo biosynthesis of vanillin in fission yeast (*Schizosaccharomyces pombe*) and baker's yeast (*Saccharomyces cerevisiae*). Appl. Environ. Microbiol. 75, 2765–2774.
14. Sukrasno, N. and Yeoman, M. M. (1993) Phenylpropanoid metabolism during growth and development of *Capsicum frutescens* fruits. Phytochemistry 32, 839.
15. Chiu, W. L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H. and Sheen, J. (1996) Engineered GFP as a vital reporter in plants. Curr. Biol. 6, 325–330.
16. Gallage, N. J. and Møller, B. L. (2015) Vanillin–bioconversion and bioengineering of the most popular plant flavor and its de novo biosynthesis in the vanilla orchid. Mol. Plant 8, 40–57.
17. Matthew, S. and Abraham, T. E. (2004) Ferulic acid: an antioxidant found naturally in plant cell walls and feruloyl esterases involved in its release and their applications. Crit. Rev. Biotechnol. 24, 59–83.
18. Dobberstein, D. and Bunzel, M. (2010) Separation and detection of cell wall-bound ferulic acid dehydrodimers and dehydrotrimers in cereals and other plant materials by reversed phase high-performance liquid chromatography with ultraviolet detection. J. Agric. Food Chem. 58, 8927–8935.
19. Yahiaoui, N., Marque, C., Myton, K. E., Negrel, J. and Boudet, A. M. (1998) Impact of different levels of cinnamyl alcohol dehydrogenase down-regulation on lignins of transgenic tobacco plants. Planta 204, 8–15.
20. Gururaj, H. B., Padma, M. N., Giridhar, P. and Ravishankar, G. A. (2012) Functional validation of *Capsicum frutescens* aminotransferase gene involved in vanillylamine biosynthesis using *Agrobacterium* mediated genetic transformation studies in *Nicotiana tabacum* and *Capsicum frutescens* calli cultures. Plant Sci. 195, 96–105.
21. Rodas-Junco, B. A., Cab-Guillén, Y., Muñoz-Sánchez, J. A., Vázquez-Flota, F., Monforte-González, M. and Hernández-Sotomayor, S. M. T. (2013) Salicylic acid induces vanillin synthesis through the phospholipid signaling pathway in *Capsicum chinense* cell cultures. Plant Signal. Behav. 8, e26752.

22. Zhang, X. and Liu, C-J. (2015) Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids. Mol. Plant 8, 17–27.
23. Pilate, G., Dejardin, A. and Leple J-C. (2010) Field trials with lignin-modified transgenic trees. In Jouanin, L. and Lapierre, C. (eds.) Lignins: Biosynthesis, Biodegradation and Bioengineering. Academic Press, USA.
24. Harris, P. J. and Trethewey, J. A. K. (2010) The distribution of ester-linked ferulic acid in the cell walls of angiosperms. Phytochem. Rev. 9, 19 –33.

7. Figure Captions

Fig. 1 The pathway to capsaicin biosynthesis from two branches: (i) via CoA derivatives of amino acid, such as valine, contributing the fatty acid moiety; and (ii) via the phenylpropanoid biosynthesis, followed by steps from ferulate to vanillylamine and subsequently capsaicin, which is unique to *Capsicum* [14]. Pathway diagram was generated by MetaCyc (http://metacyc.org/cytoscape-js/ovsubset.html?orgid=META&pwys=PWY-5710).

Fig. 2 Recombinant pcDNA6.2::(35Sp-*VpVAN*-V5)Δ*ccd*B expression vector cloned with 35Sp-*VpVAN*-V5 gene cassette after double restriction digest by *Pst*I and *Bam*HI endonucleases and ligation by T4 DNA ligase. The insertion of the gene cassette replaced a negative selectable marker, a lethal cytotoxic *ccd*B gene, that was originally present downstream of the chloramphenicol resistance gene (Cm^R). Non-resistant *E. coli* that was transformed with recircularized (non-recombinant) destination vector would be killed with the expression of intact *ccd*B gene. The presence of the ampicillin resistance gene (Amp^R) and the chloramphenicol resistance gene (Cm^R) allows selection of the bacterial transformants in Luria Bertani agar containing ampicillin and chloramphenicol to maintain the integrity of the vector. Plant transformants would confer resistance to blasticidin in the Murashige and Skoog (MS) medium with the expression of blasticidin S deaminase gene (*BSD*). The image was modified from the vector representation diagram generated using SnapGene.

Fig. 3 Microscopy images of explants transformed with pcDNA6.2::(35Sp-*VpVAN*-V5)Δ*ccd*B that survived and proliferated into callus (A) and an explant that did not

survive (B) on blasticidin selective medium. An explant that was transformed with pcDNA6.2::(35Sp-s*GFP*)Δ*ccd*B (C) showed GFP expression under blue light fluorescence in the dark (D). Scale bar represents 1 mm.

Fig. 4 PCR of *VpVAN* gene from the genomic DNA extracted from transformed calli. The gene was detected in all of the callus transformants, hence indicating successful integration of the gene cassette. M – 1kb DNA ladder; (+) – PCR positive control using synthesized holding vector, pIDT-AMP:35Sp-*VpVAN*-V5, as the template; (-) – no template PCR control.

Fig. 5 PCR of *VpVAN* gene from the genomic DNA extracted from leaf tissue of *V. planifolia* and from callus of *C. frutescens*. The gene was detected in *V. planifolia*, but not in non-transformed *C. frutescens*. M – 1kb DNA ladder; (-) – no template PCR control.

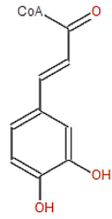
Fig. 6 HPLC chromatograms of the phenolic compounds extracted from transformed callus of *C. frutescens*. UV absorbance at 260 nm (A), 270 nm (B), 280 nm (C), and 325 nm(D) wavelengths were measured for vanillic acid, vanillin-β-D-glucoside, vanillin, and ferulic acid, respectively. Retention times for compounds in chromatograms A–D were compared with those of the standards (E) measured at 280 nmwavelength.

Fig. 7 Concentrations of vanillic acid, vanillin, ferulic acid and vanillin-β-D-glucoside, in (i) three different callus tissues after transformation with pcDNA6.2::(35Sp-*VpVAN*-V5)Δ*ccd*B expression vector (pc-VAN), and (ii) two different callus tissues that were not transformed (negative). UV absorbance was detected by HPLC at 260 nm wavelength for vanillic acid, 270 nm wavelength for vanillin-β-D-glucoside, 280 nm wavelength for vanillin, and 325 nm wavelength for ferulic acid.

Fig. S1 Representation of colony PCR of *VpVAN* gene from thirteen *E. coli* colonies transformed with pcDNA6.2::(35Sp-*VpVAN*-V5)Δ*ccd*B expression vector. M – 1kb DNA ladder; (+) – PCR positive control using synthesized holding vector, pIDT-AMP:35Sp-*VpVAN*-V5, as the template; (-) – no template PCR control.

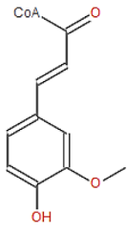
Fig. S2 (i) PCR of *VpVAN* gene from pcDNA6.2::(35Sp-*VpVAN*-V5)Δ*ccd*B expression vector extracted from *E. coli*, and (ii) DNA profile of the extracted expression vector after double restriction digest with *Pst*I and *Bam*HI restriction

endonucleases (REs). M – 1kb DNA ladder; (+) – PCR positive control using synthesized holding vector, pIDT-AMP:35Sp-*VpVAN*-V5, as the template; (-) – no template PCR control.



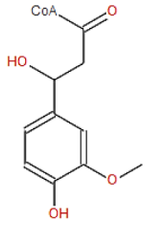
trans-caffeoyl-CoA

caffeoyl-CoA-O-methyltransferase (Cc): CCoAOMT



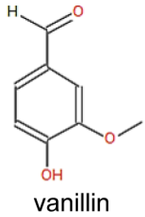
feruloyl-CoA

4-hydroxy-3-methoxyphenyl-hydroxypropanoyl-CoA hydrolyase (feruloyl-CoA-forming) (C sp.): HCHL



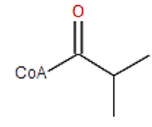
4-hydroxyl-3-methoxyphenyl-β-hydroxypropanoyl-CoA

3-hydroxy-3-(4-hydroxyl-3-methoxyphenyl) propanoyl-CoA vanillin-lyase (acetyl-CoA-forming) (C sp.): HCHL



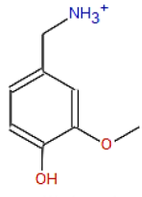
vanillin

vanillin aminotransferase (Cc): pAMT

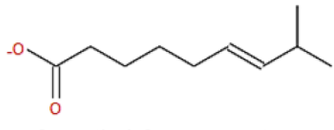


isobutanoyl-CoA

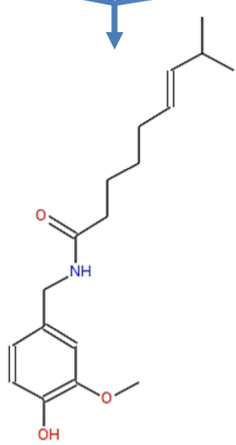
fatty acid synthase (Cc): Fat Kas Acl1



vanillylamine



8-methyl-6-nonenoate



capsaicin

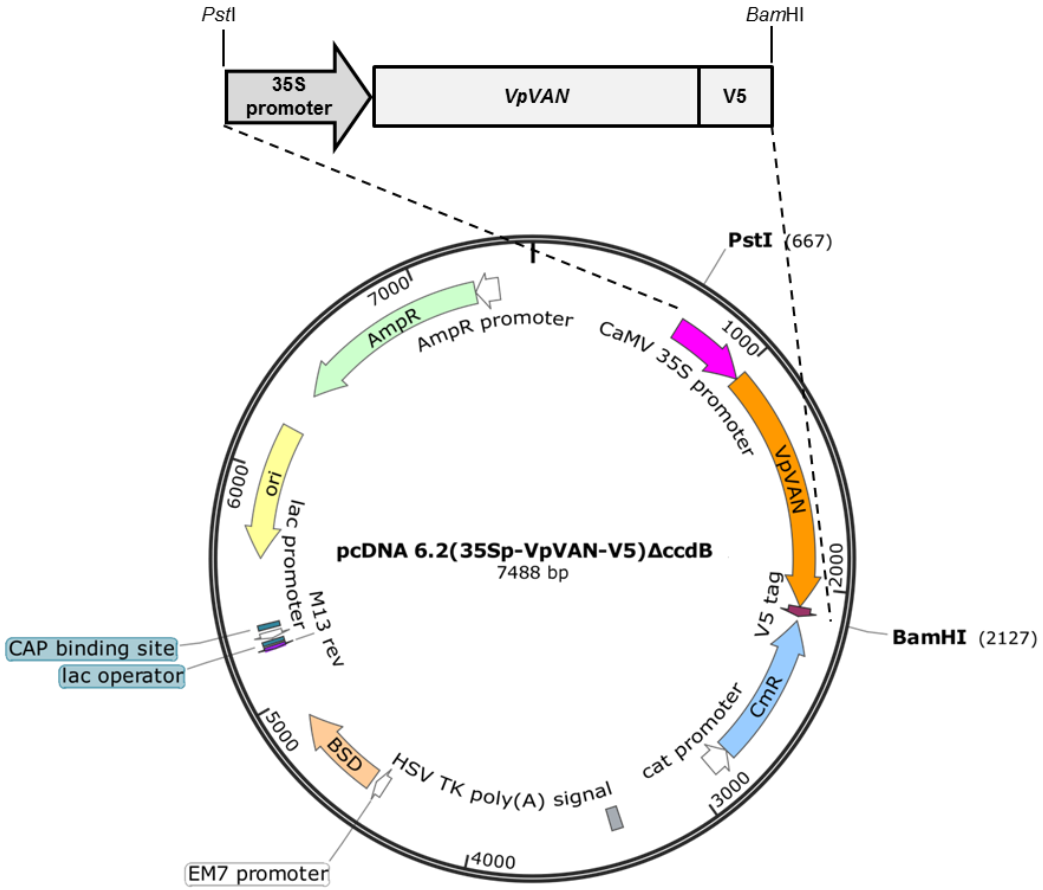
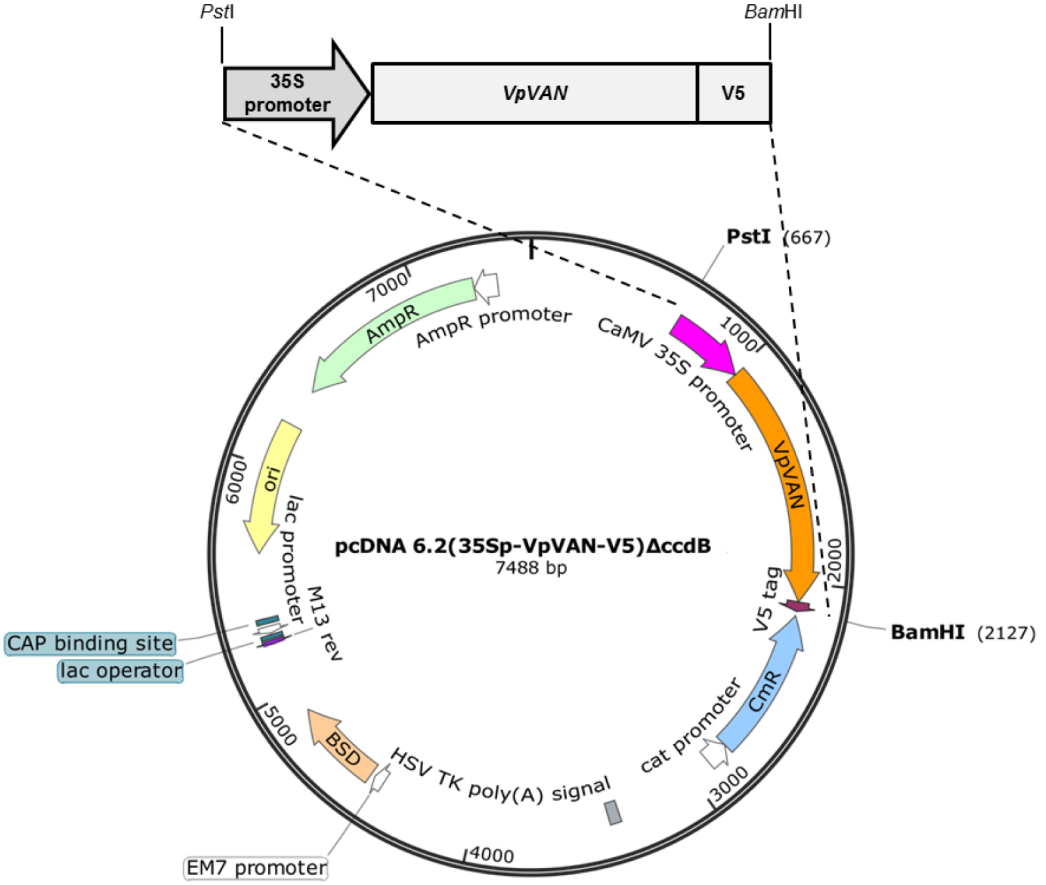
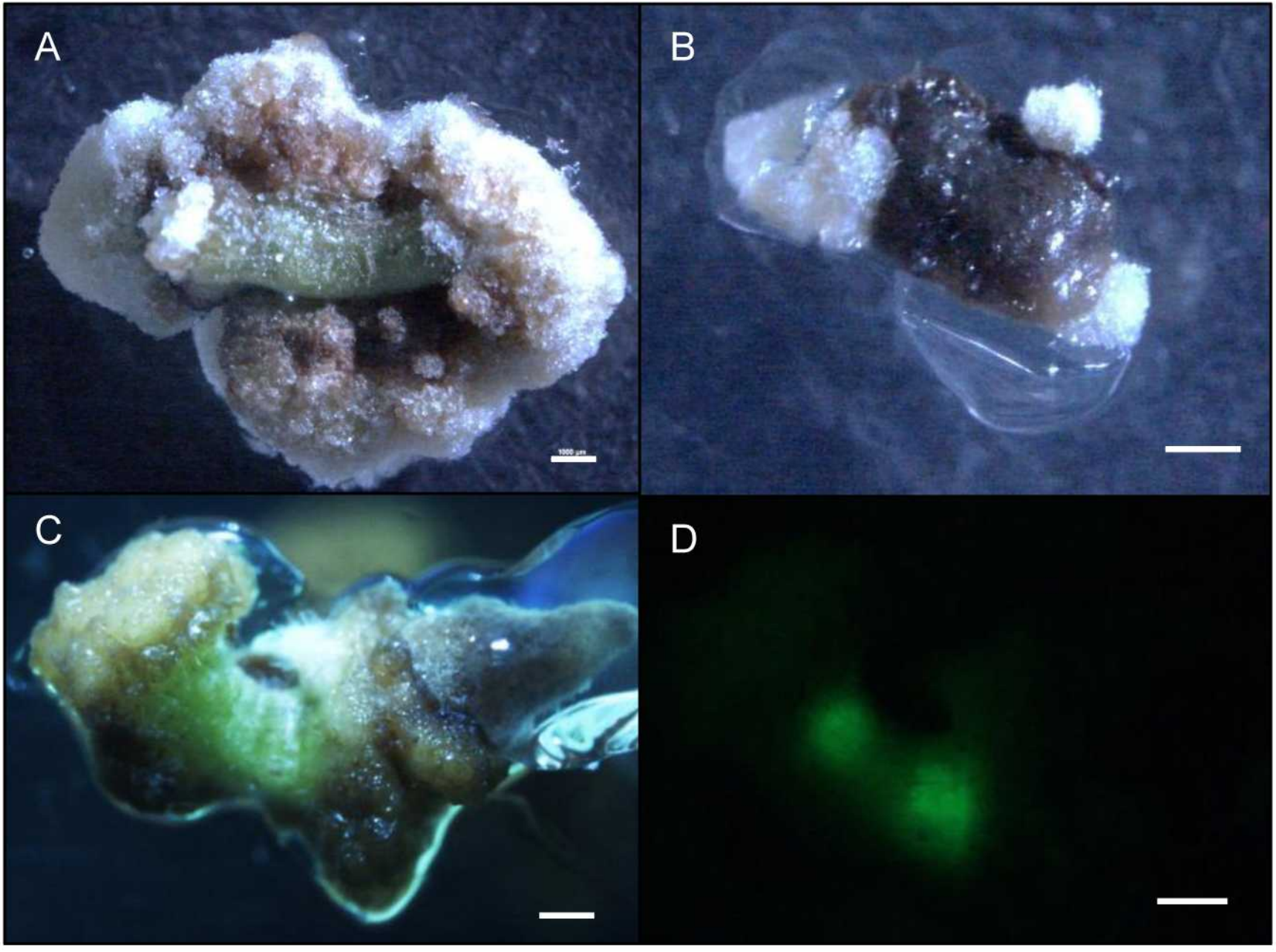
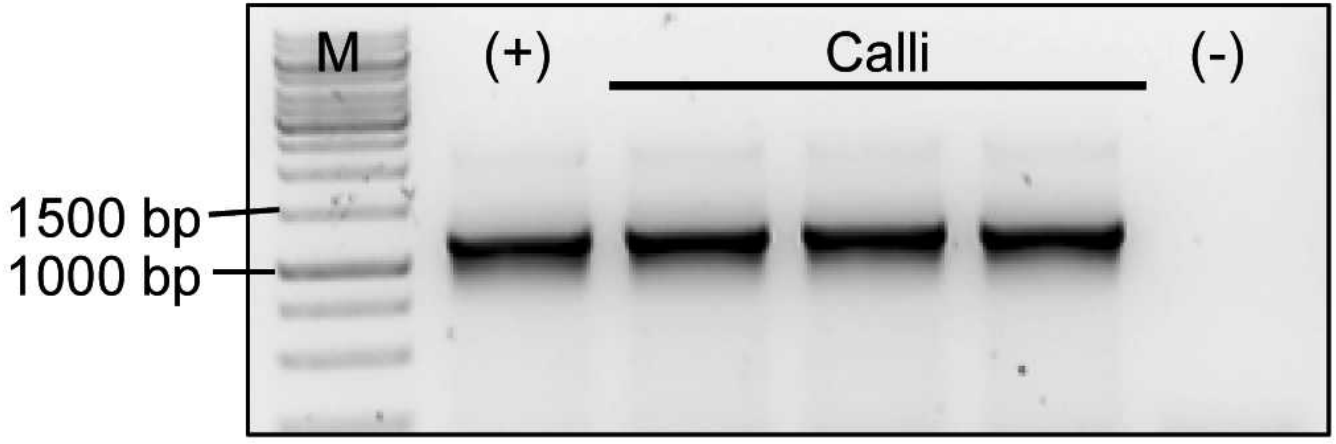
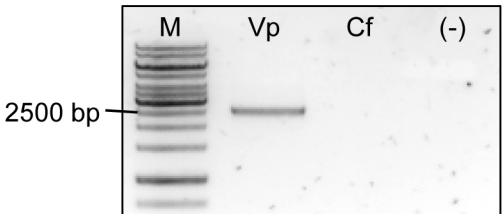


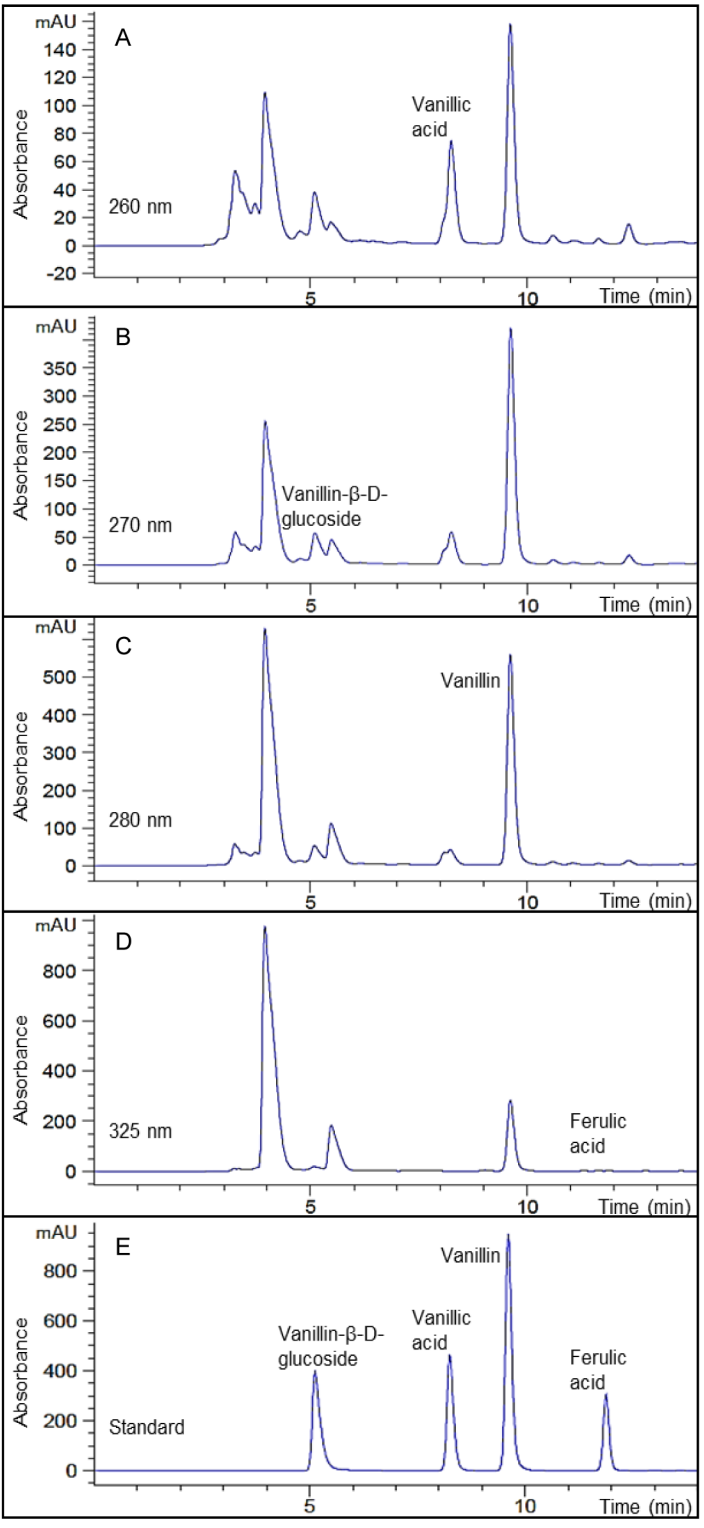
Figure 2 Recombinant pcDNA6.2::(35Sp-*VpVAN*-V5)Δ*ccd*B expression vector cloned with 35Sp-*VpVAN*-V5 gene cassette after double restriction digest by *Pst*I and *Bam*HI endonucleases and ligation by T4 DNA ligase. The insertion of the gene cassette replaced a negative selectable marker, a lethal cytotoxic *ccd*B gene, that was originally present downstream of the chloramphenicol resistance gene (Cm^R). Non-resistant *E. coli* that was transformed with recircularized (non-recombinant) destination vector would be killed with the expression of intact *ccd*B gene. The presence of the ampicillin resistance gene (Amp^R) and the chloramphenicol resistance gene (Cm^R) allows selection of the bacterial transformants in Luria Bertani agar containing ampicillin and chloramphenicol to maintain the integrity of the vector. Plant transformants would confer resistance to blasticidin in the Murashige and Skoog (MS) medium with the expression of blasticidin S deaminase gene (*BSD*). Image above was modified from the vector representation diagram generated using SnapGene.

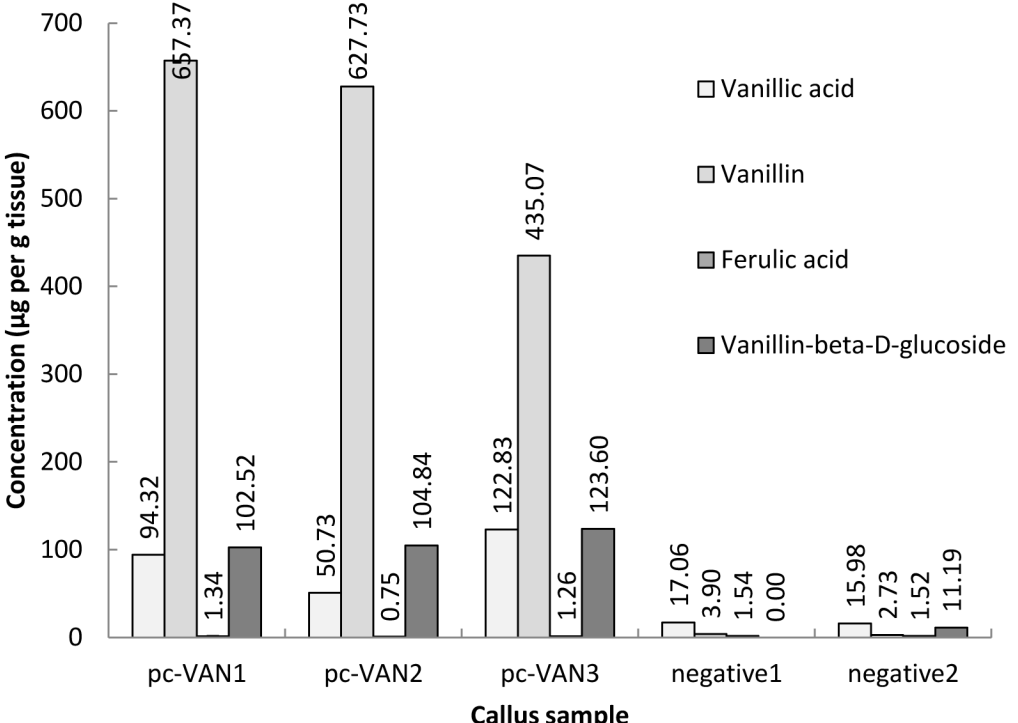

PstI
BamHI
35S promoter
VpVAN
V5
PstI (667)
BamHI (2127)
pcDNA 6.2(35Sp-VpVAN-V5)ΔccdB
7488 bp
AmpR
AmpR promoter
CaMV 35S promoter
VpVAN
V5 tag
CmR
cat promoter
HSV TK poly(A) signal
BSD
EM7 promoter
M13 rev
lac promoter
ori
CAP binding site
lac operator
1000
2000
3000
4000
5000
6000
7000

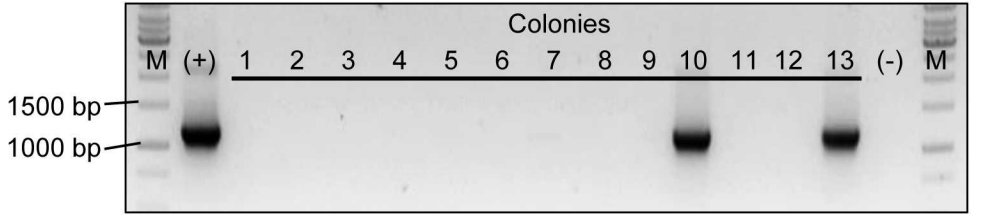

A
B
C
D

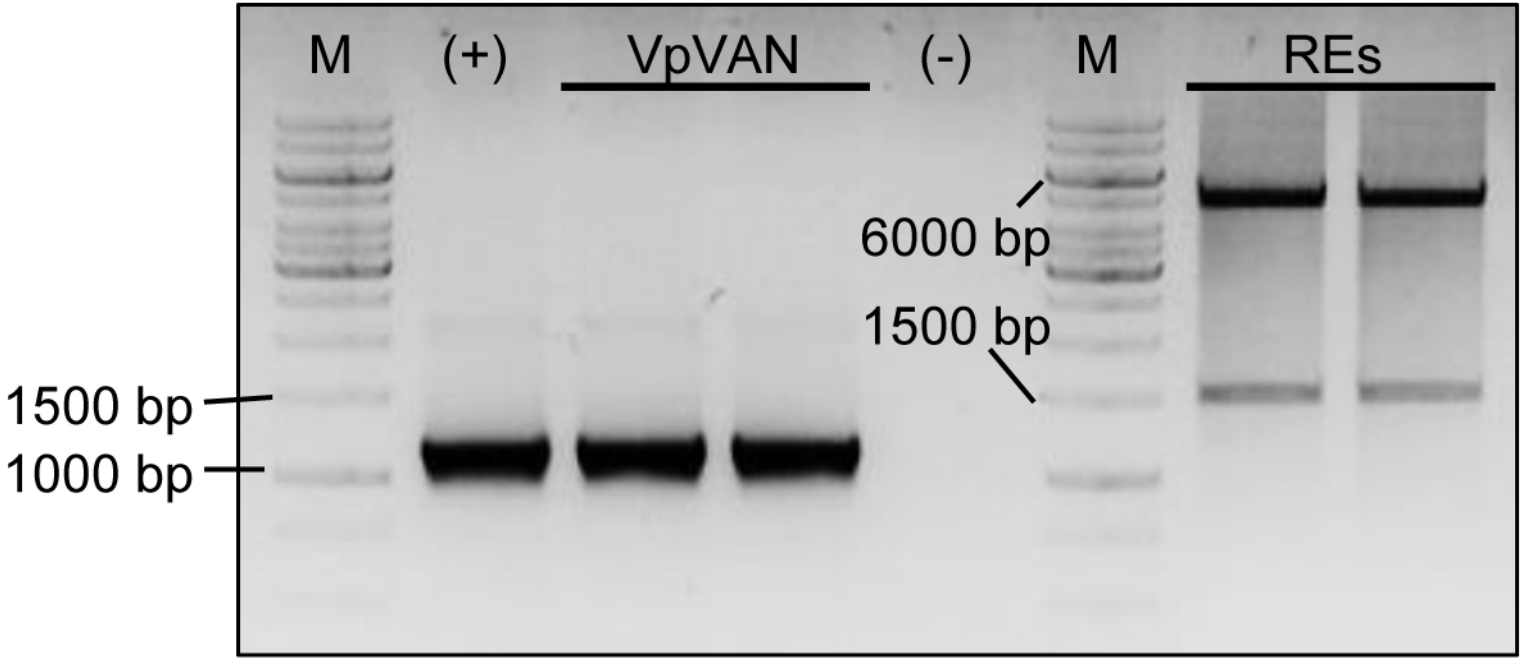

M
(+)
Calli
(-)
1500 bp
1000 bp


M
Vp
Cf
(-)
2500 bp


A
mAU
Absorbance
260 nm
Vanillic acid
Time (min)
B
mAU
Absorbance
270 nm
Vanillin-β-D-glucoside
Time (min)
C
mAU
Absorbance
280 nm
Vanillin
Time (min)
D
mAU
Absorbance
325 nm
Ferulic acid
Time (min)
E
mAU
Absorbance
Standard
Vanillin-β-D-glucoside
Vanillic acid
Vanillin
Ferulic acid
Time (min)


700
600
500
400
300
200
100
0
Concentration (µg per g tissue)
Vanillic acid
Vanillin
Ferulic acid
Vanillin-beta-D-glucoside
94.32
657.37
1.34
102.52
50.73
627.73
0.75
104.84
122.83
435.07
1.26
123.60
17.06
3.90
1.54
0.00
15.98
2.73
1.52
11.19
pc-VAN1
pc-VAN2
pc-VAN3
negative1
negative2
Callus sample


Colonies
M
(+)
1
2
3
4
5
6
7
8
9
10
11
12
13
(-)
M
1500 bp
1000 bp


M
(+)
VpVAN
(-)
M
REs
6000 bp
1500 bp
1500 bp
1000 bp