Taylor & Francis
Taylor & Francis Group

Journal of Biomolecular Structure and Dynamics

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tbsd20

In silico assisted identification of peppery aroma compound 'rotundone' backbone genes from black pepper

Johnson George Kokkat, Sreekumar Shelvy, Abdulkabeer Muhammed Fayad, Ahammed Shabeer T. P., Palaniyandi Umadevi, Rajaram Kale, Ulavappa Basavanneppa Angadi, Mir Asif Iquebal, Sarika Jaiswal, Anil Rai & Dinesh Kumar

To cite this article: Johnson George Kokkat, Sreekumar Shelvy, Abdulkabeer Muhammed Fayad, Ahammed Shabeer T. P., Palaniyandi Umadevi, Rajaram Kale, Ulavappa Basavanneppa Angadi, Mir Asif Iquebal, Sarika Jaiswal, Anil Rai & Dinesh Kumar (2022) *In silico* assisted identification of peppery aroma compound 'rotundone' backbone genes from black pepper, Journal of Biomolecular Structure and Dynamics, 40:14, 6398-6404, DOI: 10.1080/07391102.2021.1883113

To link to this article: https://doi.org/10.1080/07391102.2021.1883113

JOURNAL OF BIOMOLECULAR STRUCTURE AND DYNAMICS
2022, VOL. 40, NO. 14, 6398–6404
https://doi.org/10.1080/07391102.2021.1883113

In silico assisted identification of peppery aroma compound 'rotundone' backbone genes from black pepper

Johnson George Kokkat[a], Sreekumar Shelvy[a], Abdulkabeer Muhammed Fayad[a], Ahammed Shabeer T. P.[b], Palaniyandi Umadevi[a,c], Rajaram Kale[b], Ulavappa Basavanneppa Angadi[d], Mir Asif Iquebal[d], Sarika Jaiswal[d], Anil Rai[d] and Dinesh Kumar[d]

[a]ICAR – Indian Institute of Spices Research, Kozhikode, India; [b]ICAR – National Research Centre for Grapes, Pune, India; [c]Rice Breeding & Genetics Research Center, ICAR –Indian Agricultural Research Institute, Aduthurai, India; [d]ICAR – Indian Agricultural Statistics Research Institute, New Delhi, India

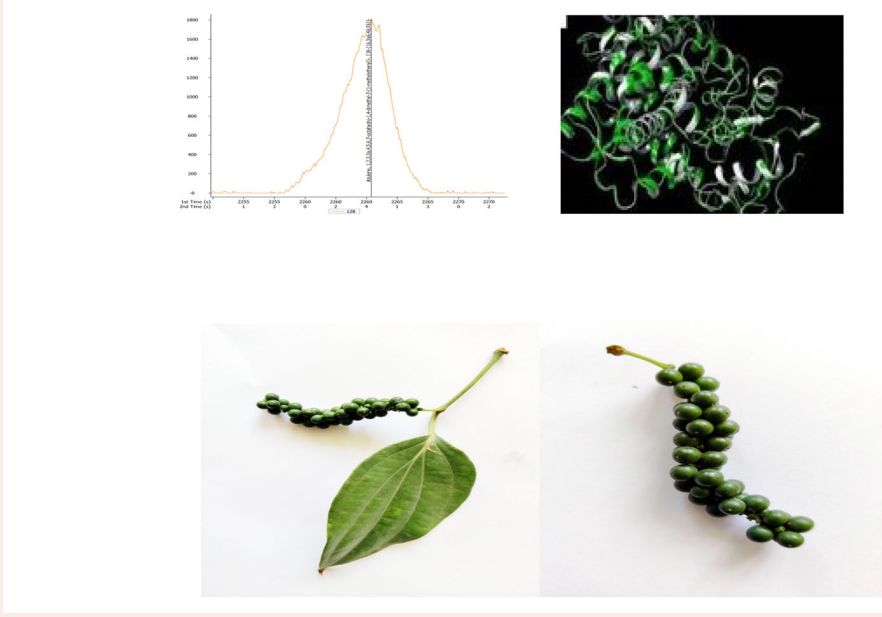
Communicated by Ramaswamy H. Sarma

ABSTRACT

Rotundone, an oxygenated sesquiterpene compound, responsible for the peppery aroma. The importance of the rotundone in the flavor industry warrants search for the precursor genes in plants. We report in this study, the first on the identification of rotundone backbone genes viz., α-guaiene synthase & α-guaiene oxidase in black pepper. We identified the precursor genes of rotundone using berry transcriptome profiling. The metabolite profiling using head space mass spectrometry showed the presence of the direct precursor compounds for rotundone biosynthesis in black pepper berries. The identification of the genes & compounds of the guaiene skeleton is expected to help in bioprospecting of black pepper varieties & also in recombinant production of the aroma compound.

ARTICLE HISTORY
Received 3 August 2020
Accepted 25 January 2021

KEYWORDS
Piper nigrum; alpha quaeine synthase; alpha quaeine oxidase; berry transcriptomemetabolite profiling



Identification of rotundone backbone genes & precursor compounds from *Piper nigrum*

1. Introduction

The black pepper berries (also called pepper fruit or peppercorn), contains various aroma-rendering mono and sesquiterpenes. Rotundone an oxygenated sesquiterpene was identified as compound responsible for the peppery aroma of Shiraz wine varieties (Wood et al., 2008). Recently, the *Vv*GuaS gene from grape berries was demonstrated to produce α-guaiene, the precursor of rotundone (Drew et al., 2016) & *Vv*STO2 as a α-guaiene 2-oxidase which transforms α-guaiene to rotundone in the grape cultivar Syrah (Takase et al., 2016). Rotundone is found in plants that also contain α-guaiene and other guaiene type sesquiterpenes (Drew

CONTACT Palaniyandi Umadevi umadevi.p@icar.gov.in; Dinesh Kumar dinesh.kumar@icar.gov.in
Supplemental data for this article can be accessed online at https://doi.org/10.1080/07391102.2021.1883113

Figure 1. Half matured berry samples of *Piper nigrum* variety IISR-Thevam.

et al., 2016). Using chromatographic methods, the α-guaiene and β-guaiene compounds were identified in black and white pepper (*Piper* spp.) (Jirovetz et al., 2002). Higher amounts of rotundone were reported in black and white pepper corn, which was ~1000 times the amount of very peppery wine and other plants tested (Wood et al., 2008). But there are no reports till now on the identification of the genes involved in the rotundone synthesis in black pepper.

Because of its potent peppery aroma, the molecular basis and genes in rotundone formation is of particular important in black pepper. The identification of gene or genes responsible for rotundone biosynthesis in black pepper would use them in bioprospecting of black pepper varieties with desirable amount of rotundone, use the genes in marker assisted selection and metabolic engineering. The rotundone backbone genes in black pepper unripe pepper corn from previous transcriptome based studies (Jin et al., 2018) still remains undiscovered. In light of this, we attempted the identification of rotundone back bone genes of black pepper from the berry transcriptome developed by us (Bio Sample accession: SAMN13981803). We identified the α-guaiene synthase & α-guaiene oxidase genes in the transcriptome. Furthermore, we showed the expression levels in two tissues and phylogeny of the identified genes. The identification of rotundone precursor compounds from the metabolite profiling of berries using GCxGC Time-Of-Flight Mass Spectrometer further confirms the presence of rotundone synthesis in black pepper.

2. Results

2.1. Transcriptome mining for PnAGS & PnAGO genes

De novo hybrid transcriptome assembly of half matured berry samples of IISR-Thevam was done using Illumina and Nanopore sequencing data. The Illumina sequencing generated a total of 38,153,296 (38.1 million) raw reads. The *de novo* assembly produced 308,369 contigs with an average contig length of 1119 bp and a maximum length of 27,769 bp. This hybrid transcriptome assembly was used in the present study for the identification of rotundone synthesis backbone genes.

The tblastn resulted 115 contigs from the transcriptome that are similar to the seed sequences transcripts with highest bits score was selected for further analysis. The blast analysis for *Pn*AGS showed 42.33% similarity with characterized *Vv*GuaS gene. The *Pn*AGO showed 51.96% similarity with characterized *Vv*STO2 gene. In case of *Pn*AGO the tblastn resulted 500 contigs from the transcriptome that are similar to the seed sequences. Transcripts with most bits score was selected for further analysis.

Phylogenetic analysis (Figure 2(a)) revealed the ancestral relationship among the *Pn*AGS homologues. Sesquiterpene synthase of *Piper* sps. were falling into one clade while the other homologues of AGS from *vitis* and *pogostemon* are in another group. All the α-guaiene synthase genes were clustered as one group while the δ-guaiene was distinct from the group. The phylogeny of *Pn*AGO (Figure 2(b)) showed that the Cytochrome p450 genes and *Pn*AGO were closely related. *Pn*AGO is highly closed to *Cinnamomum micranthum* Cyp450.

Through NCBI-Conserved Domain Database search, the plant terpene cyclises & the terpene synthase metal binding domains were identified in the *Pn*AGS. The *Pn*AGO showed the p450 superfamily conserved domain (Supplementary File)

The Interproscan analysis also revealed the protein signature recognitions of *Pn*AGS and *Pn*AGO. The analysis confirmed the relationship of *Pn*AGS to the superfamily of plant terpene cyclases and Terpene synthase metal binding and *Pn*AGO to the Cytochrome p450 superfamily (Supplementary File)

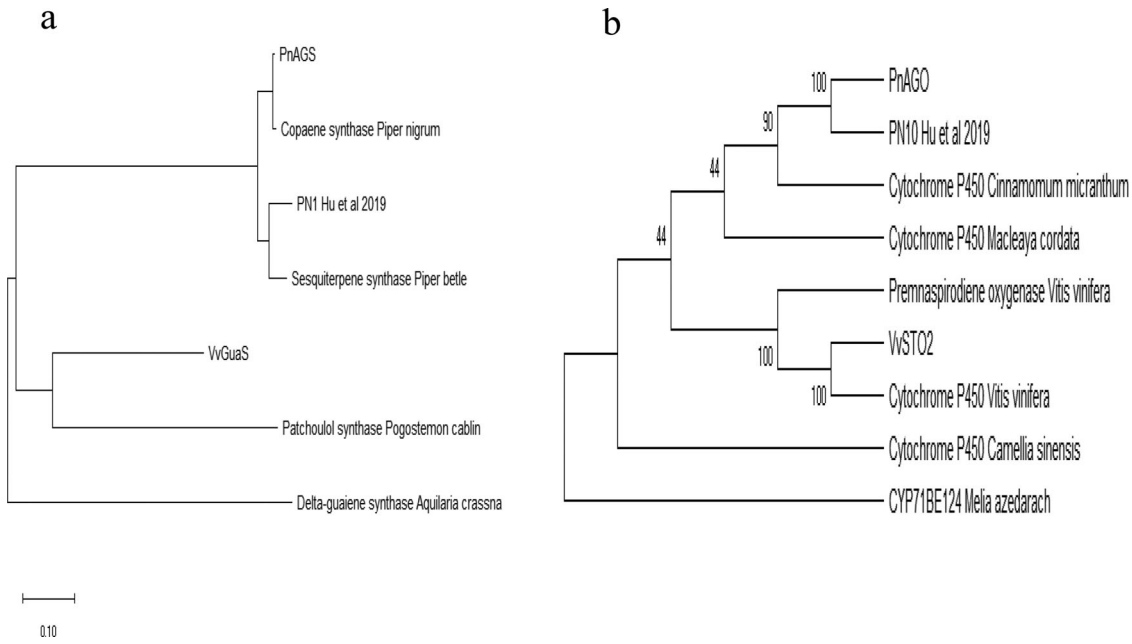


Figure 2. (a) Phylogenetic analysis of *Pn*AGS, (b) Phylogenetic analysis of *Pn*AGO.

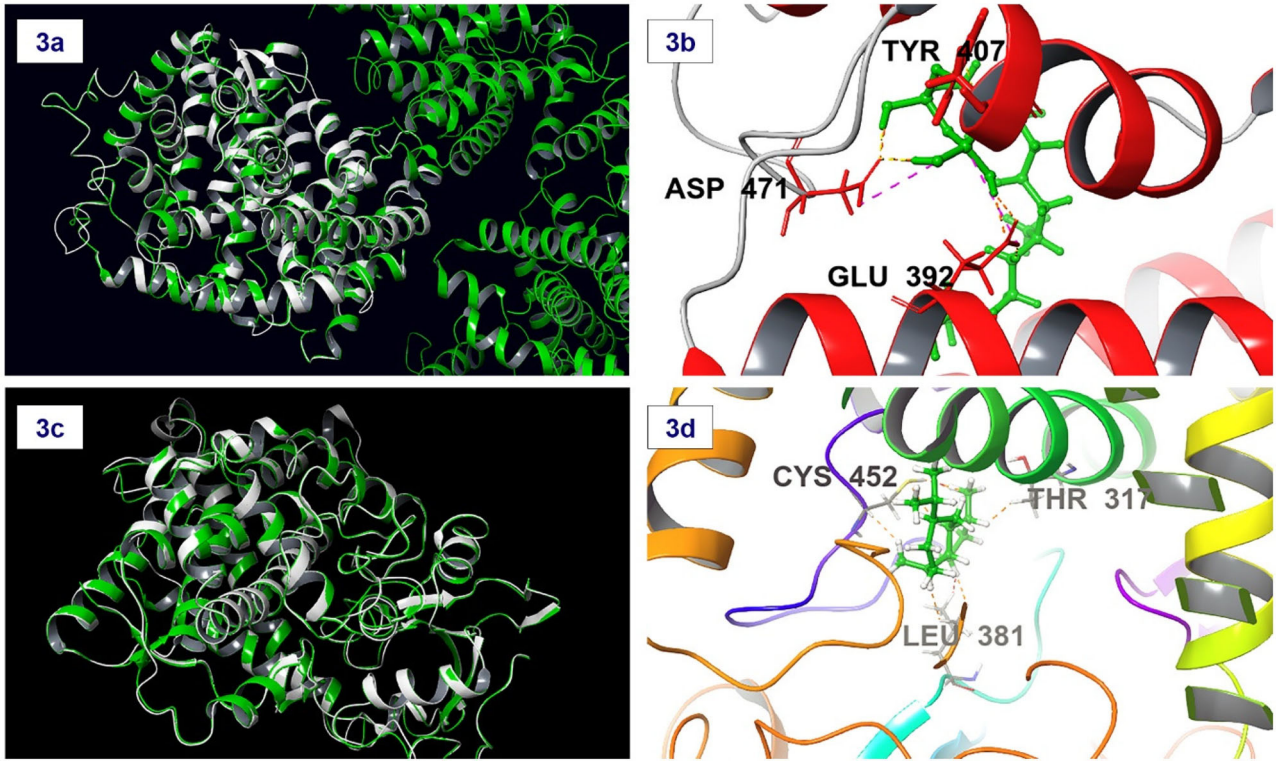


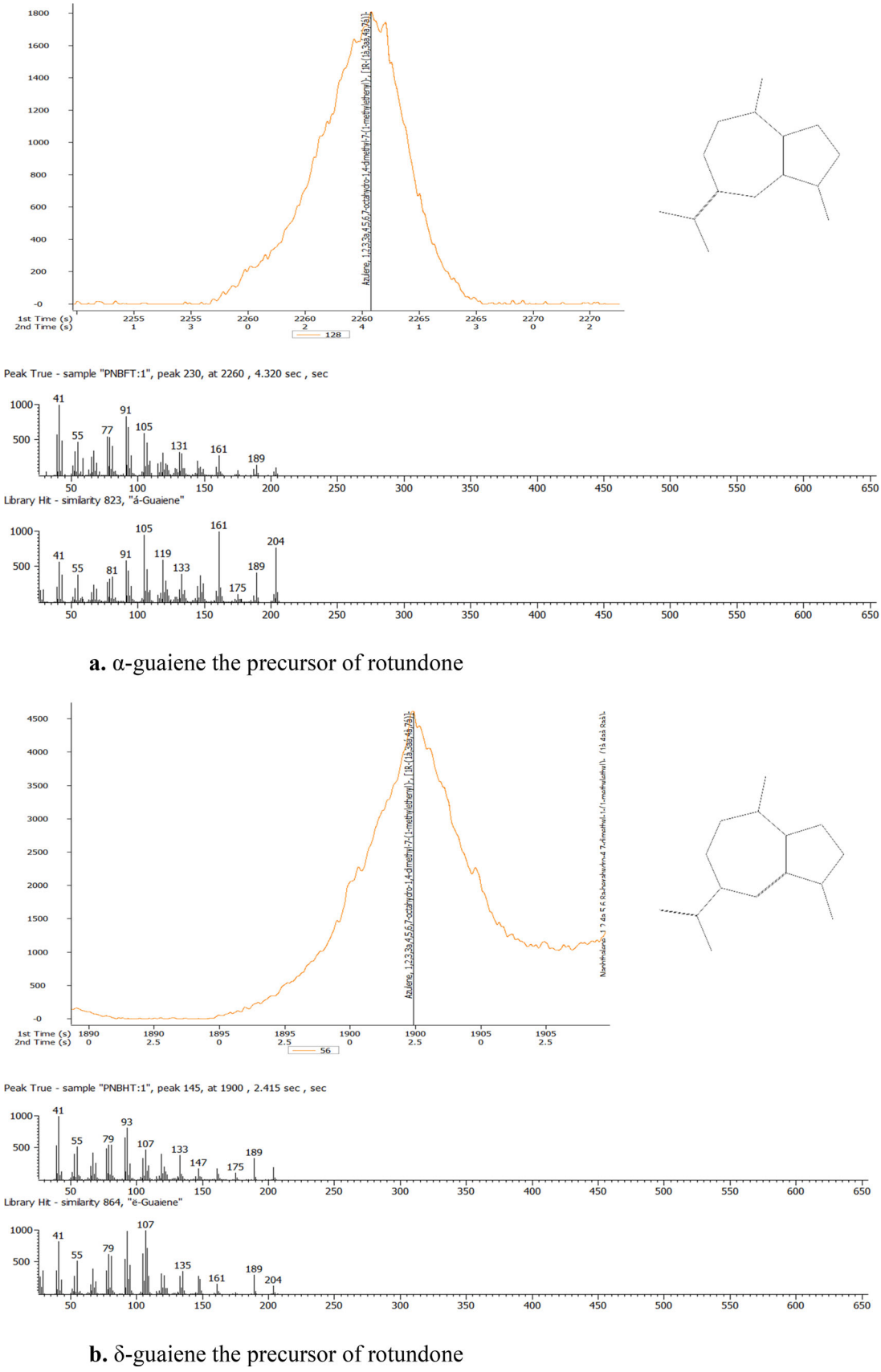
Figure 3. (a) Structure of *Pn*AGS (grey) with 5JO7 (green), (b) FPP in active site (ASP471, TYR407, GLU392), (c) Structure of *Pn*AGO (grey) with 5YLW (green), (d) α-guaiene in active site (CYS 452, LEU 381, THR 317).

2.2. Homology modelling

The homology modelling was done to find the amino acid residues present near the FPP binding site as the difference in the binding site amino acid determines the nature of metabolite produced by the sesquiterpene synthase enzymes (Greenhagen et al., 2006). The AGS had ASP471, TYR407, GLU392 amino acids in their FPP biding site (Figure 3(a,b)). In case of *Pn*AGO, the homology modelling revealed that THR 317, LEU381, CYS452 as the active site binding amino acid residues in contrast to this *Vitis vinifera* guaiene oxidase (*Vv*STO2) showed 35.50% similarity with the same template (Figure 3(c,d)).

2.3. Quantitative real-time PCR

Real-time PCR analysis was depicted through relative quantification study to calculate the fold expression of AGS and AGO in leaf and berry. Analysis was done using cDNA from



a. α-guaiene the precursor of rotundone

b. δ-guaiene the precursor of rotundone

Figure 4. (a) α-guaiene the precursor of rotundone. (b) δ-guaiene the precursor of rotundone.

leaf (AGS Avg. Ct – 37.752) and (AGO Avg. Ct – 32.361) and half matured berry (AGS Avg. Ct – 33.511) and (AGO Avg. Ct – 32.412). Normalized using the Ubiquitin levels by the formula $dC_T = dC_T$ (AGS/AGO) - dC_T (Ubiquitin) where the average values of ubiquitin are Avg. AGS Ct – 31.820 and AGO Ct – 29.328. The AGS showed upregulation in leaf

while the AGO showed least expression. The expression of both the genes were almost same in the berries. The blast analysis of sequenced PCR products of *Pn*AGS and *Pn*AGO confirmed their identity.

2.4. Metabolite profiling

To correlate the presence of the rotundone back bone genes with the precursor compounds the metabolite profiling was done with the unripe peppercorn and leaf of two varieties of Piper by head space GC-MS. Comparison of mass spectra with the MS libraries allowed the identification of the major precursor compounds of rotundone viz., α-guaiene & δ-guaiene in the berries.The compound α-guaiene (Azulene, 1,2,3,3a,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1R-(1α,3aβ,4α,7β)]-) with the retention index 1502 & retention time of 2260s was found in primary and 4.320 s in secondary column. The unique mass is 128 and the percent similarity match with NIST is 82.3 as spectra (Figure 4(a)). In the case of δ-guaiene (Azulene, 1,2,3,5,6,7,8, 8a-octahydro-1,4-dimethyl-7- (1-methylethenyl)-, [1S-(1α,7α,8αβ)]-). The retention index was 1655 & retention time 1900s in primary and 2.415 s in secondary column was found. The unique mass is 56 and the percent similarity match with NIST is 86.4 as shown in spectra (Figure 4(b)).

3. Discussion

The earlier fruit transcriptomes (Hu et al., 2015; Jin et al., 2018) described only the genes involved in Piperine synthesis and other terpene synthases. The rotundone backbone genes in black pepper unripe pepper corn from previous transcriptome based studies (Jin et al., 2018) still remains undiscovered. They attributed the absence of the genes to that of extremely low abundance of the transcript for α-guaiene biosynthesis in peppercorn and cultivar specific expression of the target genes in the genotype used for metabolite and transcript profiling in their work. In the present study, the identification of 115 & 500 contigs similar to the AGS & AGO seed sequences transcripts, respectively, showed its presence in the hybrid transcriptome. The PnAGS phylogeny also placed this gene in sesquiterpene synthase of *Piper* sps. clade and distanced it from the δ-guaiene group. In the same way, PnAGO was placed in Cytochrome p450 gene clade. Along with the phylogeny the NCBI-Conserved Domain Database search & Interproscan also added the confirmation as *Pn*AGS had the plant terpene cyclases & the terpene synthase metal binding domains & the *Pn*AGO with the p450 superfamily conserved domain. Hence, the present study showed the presence of the *Pn*AGS and *Pn*AGO transcripts for the first time from a fruit trascriptome of black pepper & it is expected that this hybrid transcriptome would be a valuable resource for characterising important genes from Piper species. The real time expression and the validation of the identity using sequencing of the PCR product showed the active backbone genes of peppery aroma rotundone in our variety IISR-Thevam and IISR-Shakthi. Although Jin et al. (2018) stated that the presence of rotundone backbone genes are cultivar specific, the present study showed the expression of AGS & AGO genes in two important black pepper varieties. The genome wide analysis using reference genome (Hu et al., 2019) also evidenced the genes in black pepper with PnAGS & PnAGO in Pseudochromosome PN1 & PN10, respectively.

The template 5-epi-aristolochene synthase was used for homology modelling of α-guaiene synthase gene (*Vv*GuaS) of *Vitis*. The amino acid residues T414 & V530 were found to be present in binding site to FPP (Drew et al., 2016). The amino acid ASP471, TYR407, GLU392 were found to be present in the *Pn*AGS gene to the FPP binding when the template Vetispiradiene synthase 5JO7 was used which suggest that these two may be the variant of AGS. The amino acid sequence of *Pn*AGS and *Vv*GuaS shared 42.73 percentage of similarity with each other.

The *Pn*AGS transcript level was relatively less in berries when compared to leaf. Jin et al. (2018) also showed the low transcript level of three important sesquiterpene synthases (*Pn*TPS) of *Piper* in roots and fruits compared to stem and leaves. The *Pn*AGO transcript level was higher in berry, when compared to leaf. This might be due to the major oxidation of α-guaiene to rotundone by AGO in berries than leaf. The transcript levels of both *Pn*AGS and *Pn*AGO were of same in berry. This result is correlating well with the detailed analysis of rotundone in pepper corn by Wood et al. (2008). In grapes also, although the rotundone is identified in various plant parts, the occurrence was more in fruit. Since we analyzed the expression of *Pn*AGS and *Pn*AGO in only half matured berries, we could not correlate the expression levels of these genes at varying levels of maturity. Analysis focusing the different developmental stages of berry and different varieties would provide more information on the dynamics of the 'peppery flavor' marker genes in this crop.

The head space GC-MS analysis of different developmental stages of peppercorns, including early stage and fully ripened peppercorns showed no α-guaiene-type sesquiterpenes (Jeleń & Gracka, 2015).Whereas the present study identified the precursor molecules (α-guaiene and delta guaiene) of rotundone synthesis in full and half matured berries of variety IISR-Shakthi. The black pepper variety IISR–Thevam showed the presence of α-gurjunene which is also demonstrated to exhibit the characteristic guaiene-type 5, 7 bicyclic carbon skeleton of the peppery aroma compound rotundone (Drew et al., 2016). The presence of unique guaiene-type sesquiterpene in the tested varieties may be due to the cultivar specific nature of these products. The WT *Vv*GuaS enzyme of grapes was also found to encode four major products of the guaiene skeleton viz., α-guaiene, α-bulnesene, epiglobulol and γ-gurjunene.

To conclude, the *in silico* assisted approach identified the precursor genes of rotundone using transcript profiling. The metabolite profiling ensured the presence of the direct precursor compounds for rotundone biosynthesis in black pepper. The information on the gene sequence, expression pattern will help in developing functional markers for use in MAS in black pepper and also in recombinant production of the aroma compound. The identification of the compounds

of guaiene skeleton would help in bringing out elite chemotypes towards bioprospecting of black pepper varieties for aroma industries.

4. Materials and methods

4.1. Berry transcriptome

Half matured berry samples of *P. nigrum* variety IISR-Thevam were collected from ICAR – Indian Institute of Spices Research experimental farm, Kozhikode, Kerala (Figure 1). The total RNA was extracted by modified Spectrum Plant Total RNA Kit (STRN50-Sigma) protocol. RNA integrity was evaluated using Bioanalyzer 2100 (Agilent, USA). Samples with RNA integrity number higher than 6 were used for sequencing. *De novo* transcriptome sequencing of berry samples was done by Illumina and Nanopore sequencing. The Illumina data were demultiplexing using bcl2fastq and nanopore fast5 data were base-called using Albacore. The quality of the Illumina data was analyzed using FastQC. The short reads were processed to velvet *de novo* assembly. Data from both Illumina and Nanopore platforms and a short-read transcriptome assembly was submitted to a hybrid transcriptome assembly using IDP-*denovo* Assembler.

4.2. Identification of rotundone synthesis genes

Homologs of α-guaiene Synthase (AGS): *Pogostemon cablin* - Q49SP3, *Aquilaria crassna* - D0VMR6, *Aquilaria malaccensiswere* - A0A2L1K150, *Striga asiatica* - A0A5A7PMW7 and α-guaiene 2-oxidase (AGO): *Vitis vinefera* - F6I534, A0A438K0K2 & A0A438EQZ5 were retrieved from Uniprot. These amino acid sequences were used as the query sequence for tblastn. The *P. nigrum* transcriptome (Bio Sample accession: SAMN13981803) were used as the local nucleotide database for the identification of AGS and AGO in *Piper*. The most matching sequence with the *E*-value cut of e^{-10} were selected for further analysis.

4.3. Phylogenetic analysis

Phylogenetic analysis for *Pn*AGS was performed with the following accessions with the highest similarity (AVY53326.1, ANA50340.1) from blastp, predicted *Vv*GuaS amino acid sequences of *V. vinifera,* most matching sequence from *Piper nigrum* whole genome (Hu et al., 2019) against the query sequence and two identified AGS sequences (Q49SP3 and D0VMR6) from Uniprot. *Pn*AGO was performed with the sequences from highest similarity accessions XP_010644547.1, OVA07937.1, RWR72824.1, RVX14742.1, XP_028121781.1 and QDZ36310.1 from blastp, predicted *Vv*STO2 amino acid sequences of *V. vinifera,* most matching sequence from *Piper nigrum* reference genome (Hu et al., 2019) against the query sequence. The sequence alignment and phylogenetic tree were done using MEGA 10.0.5 and the parameters used in clustal alignment are 10 for gap opening penalty and 0.1 for gap extension penalty in pairwise alignment; 10 for gap opening penalty and 0.2 for gap penalty in multiple alignment. The maximum likelihood tree was constructed using 500 bootstrap tests with Poisson model and other uniform rates.

4.4. Domain search

Domains that are present in the target sequences were analysed using NCBI Conserved Domain Search database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and InterPro (https://www.ebi.ac.uk/interpro/search/sequence/) for identifying the protein families.

4.5. Homology modelling

The template Vetispiradiene synthase 5JO7 with 40.65% identity was retrieved from PDB and Homology modelling of PnAGS was done in SWISS MODEL Workspace. The structural analogue of FPP was included in the template structure to find the residues present in the binding site of *Pn*AGS. For *Pn*AGO the template Ferruginol synthase 5YLW with 32.46% identity, structural analogue of α-guaiene were used for the modelling SWISS MODEL workspace (Waterhouse et al., 2018).

4.6. Primer design

The primers were designed using Primer 3 plus software from the unigenes *Pn*AGS & *Pn*AGO for the rel time expression analysis.

4.7. Quantitative real-time PCR

RNA was extracted from black pepper variety IISR-Thevam (Half matured berry and leaf) by Spectrum Plant Total RNA Kit (Sigma Aldrich, USA) modified protocol. The cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (Thermo fisher, USA). Transcript levels of *Pn*AGS & *Pn*AGO were determined by qRT-PCR using primer pairs 5′-GACACAAGTCAGGCAGGTATAA-3′(F), 5′-TCAGTGGAATCTCTTCCTTTGG-3′(R) and 5′-GGAGCTTGCGACATATCCTTAT-3′ (F), 5′-GTGGAACGACTCTATCCTCTTG-3′ (R), respectively. The PCR in a total reaction volume of 20 µL containing 10 µL of 2× Rotor-Gene SYBR® Green PCR Kit (Qiagen, Germany), The PCR reaction was run on Rotor-Gene 2000 Real Time Cycler (Qiagen, Germany) with a temperature program of 5 min at 95 °C, 40 cycles of 10 s at 95 °C, 30 s at 60 °C and 20 s at 72 °C. For each sample, three replications were kept for the analysis. The Ct values were calculated. Ubiquitin gene 5′-CGTGGAGGAATGCAGATTTT-3′(F), 5′-GACACCTTCAACGCCATTTAC-3′(R) was used as the internal control.

4.8. Verification of amplified products

The qRT-PCR products were separated on a 2% agarose gel and purified using GenElute™ Gel Extraction Kit (Sigma Aldrich, USA) and sequenced in order to verify their identity. A local BLAST search was done with the *Piper* transcriptome to match the sequences to the annotated transcripts, also NCBI BLAST search was performed for confirming identity.

4.9. Rotundone precursor identification using GC × GC/TOFMS

A LECO Pegasus® (USA) 4 D, GC × GC Time-Of-Flight Mass Spectrometer (GC × GC/TOFMS) was used for identification of Rotundone precursors in black pepper samples. The GC primary oven was equipped with a 30 m DB-5MS capillary column, ID of 0.25 mm and a film thickness of 0.25 mm. This was joined to smaller DB-5MS capillary column with an ID of 0.25 mm and a film thickness of 0.25 mm of which 1.8 m was coiled in the secondary oven. The injector was held at 250 °C in the splitless mode with a purge-off time of 30 s, a 22.5 mL min^{-1} split vent flow at 1 min. Ultra-high purity (UHP) helium was used as the carrier gas at a constant flow rate of 1.5 mL min^{-1}. The temperature program was 30 °C for 1 min, ramped at 3 °C min^{-1} to 250 °C and held at 250 °C for 1 min. The secondary oven program was offset by +5 °C from the primary oven program and the modulator was offset by +15 °C from the secondary oven and −80 °C chiller temperature. The MS transfer line temperature was 225 °C. The detector voltage was set at −1700 eV.

Samples for GC × GC-TOFMS method development and evaluation were prepared in equivalent amber glass vials to prevent light degradation of some compounds. All samples were incubated at 60 °C with agitation at 500 rpm for 10 min prior to extraction at 1000 rpm.

SPME fiber of 1 cm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm 23 Ga stable Flex (Sigma Aldrich, USA) was used. DVB/CAR/PDMS SPME fibers were found suitable for nontargeted analysis of volatile compounds. Fiber desorption time of 5 min. was given at 60 °C inside the agitator. The components of headspace volatiles were identified as different peaks of the chromatogram. The compounds with different mass to charge (*m/z*) ratio have different retention times and were identified based on the National Institute of Standards and Technology (NIST 2005) mass spectra library v2.0 and authentic standard compounds like n-Hexane, Tetradecane, Pentadecane and Eicosane. The compound γ-Gurjunene identity was confirmed based on the retention time, retention index (RI) with NIST (2005) library and the compounds with spectral matching value of >700 were taken for analysis

Acknowledgements

Authors are thankful to Director, ICAR-Indian Agricultural Statistics Research Institute, New Delhi, India and Director, ICAR-Indian Institute of Spice Research, Kozhikode, India for providing research facility.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Authors are thankful to Indian Council of Agricultural Research, Ministry of Agriculture and Farmers' Welfare, Govt. of India for providing financial assistance in the form of CABin grant (F. no. Agril. Edn.4-1/2013-A&P) as well as Advanced Super Computing Hub for *Omics* Knowledge in Agriculture (ASHOKA) facility at Indian Council of Agricultural Research-Indian Agricultural Statistics Research Institute, New Delhi.

ORCID

Sreekumar Shelvy http://orcid.org/0000-0002-9525-8957
Abdulkabeer Muhammed Fayad http://orcid.org/0000-0001-8000-3065
Palaniyandi Umadevi http://orcid.org/0000-0002-5414-5948

References

Drew, D. P., Andersen, T. B., Sweetman, C., Møller, B. L., Ford, C., & Simonsen, H. T. (2016). Two key polymorphisms in a newly discovered allele of the Vitis vinifera TPS24 gene are responsible for the production of the rotundone precursor α-guaiene. *Journal of Experimental Botany*, *67*(3), 799–808. https://doi.org/10.1093/jxb/erv491

Greenhagen, B. T., O'Maille, P. E., Noel, J. P., & Chappell, J. (2006). Identifying and manipulating structural determinates linking catalytic specificities in terpene synthases. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(26), 9826–9831. https://doi.org/10.1073/pnas.0601605103

Hu, L., Hao, C., Fan, R., Wu, B., Tan, L., & Wu, H. (2015). De novo assembly and characterization of fruit transcriptome in black pepper (*Piper nigrum*). *PLoS One*, *10*(6), e0129822. https://doi.org/10.1371/journal.pone.0129822

Hu, L., Xu, Z., Wang, M., Fan, R., Yuan, D., Wu, B., Wu, H., Qin, X., Yan, L., Tan, L., Sim, S., Li, W., Saski, C. A., Daniell, H., Wendel, J. F., Lindsey, K., Zhang, X., Hao, C., & Jin, S. (2019). The chromosome-scale reference genome of black pepper provides insight into piperine biosynthesis. *Nature Communications*, *10*(1), 4702. https://doi.org/10.1038/s41467-019-12607-6

Jeleń, H. H., & Gracka, A. (2015). Analysis of black pepper volatiles by solid phase microextraction-gas chromatography: A comparison of terpenes profiles with hydrodistillation. *Journal of Chromatography A*, *1418*, 200–209. https://doi.org/10.1016/j.chroma.2015.09.065

Jin, Z., Kwon, M., Lee, A. R., Ro, D. K., Wungsintaweekul, J., & Kim, S. U. (2018). Molecular cloning and functional characterization of three terpene synthases from unripe fruit of black pepper (*Piper nigrum*). *Archives of Biochemistry and Biophysics*, *638*, 35–40. https://doi.org/10.1016/j.abb.2017.12.011

Jirovetz, L., Buchbauer, G., Ngassoum, M. B., & Geissler, M. (2002). Aroma compound analysis of *Piper nigrum* and *Piper guineense* essential oils from Cameroon using solid-phase microextraction-gas chromatography, solid-phase microextraction-gas chromatography-mass spectrometry and olfactometry. *Journal of Chromatography A*, *976*(1–2), 265–275. https://doi.org/10.1016/S0021-9673(02)00376-X

Takase, H., Sasaki, K., Shinmori, H., Shinohara, A., Mochizuki, C., Kobayashi, H., Ikoma, G., Saito, H., Matsuo, H., Suzuki, S., & Takata, R. (2016). Cytochrome P450 CYP71BE5 in grapevine (Vitis vinifera) catalyzes the formation of the spicy aroma compound (-)-rotundone. *Journal of Experimental Botany*, *67*(3), 787–798. https://doi.org/10.1093/jxb/erv496

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F. T., de Beer, T. A. P., Rempfer, C., Bordoli, L., Lepore, R., & Schwede, T. (2018). SWISS-MODEL: Homology modelling of protein structures and complexes. *Nucleic Acids Research*, *46*(W1), W296–W303. https://doi.org/10.1093/nar/gky427

Wood, C., Siebert, T. E., Parker, M., Capone, D. L., Elsey, G. M., Pollnitz, A. P., Eggers, M., Meier, M., Vössing, T., Widder, S., Krammer, G., Sefton, M. A., & Herderich, M. J. (2008). From wine to pepper: Rotundone, an obscure sesquiterpene, is a potent spicy aroma compound. *Journal of Agricultural and Food Chemistry*, *56*(10), 3738–3744. https://doi.org/10.1021/jf800183k