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Research article

Expression profiles of key phenylpropanoid genes during *Vanilla planifolia* pod development reveal a positive correlation between *PAL* gene expression and vanillin biosynthesis

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ABSTRACT

In *Vanilla planifolia* pods, development of flavor precursors is dependent on the phenylpropanoid pathway. The distinctive vanilla aroma is produced by numerous phenolic compounds of which vanillin is the most important. Because of the economic importance of vanilla, vanillin biosynthetic pathways have been extensively studied but agreement has not yet been reached on the processes leading to its accumulation. In order to explore the transcriptional control exerted on these pathways, five key phenylpropanoid genes expressed during pod development were identified and their mRNA accumulation profiles were evaluated during pod development and maturation using quantitative real-time PCR. As a prerequisite for expression analysis using qRT-PCR, five potential reference genes were tested, and two genes encoding Actin and EF1 were shown to be the most stable reference genes for accurate normalization during pod development. For the first time, genes encoding a phenylalanine ammonia-lyase (*VpPAL1*) and a cinnamate 4-hydroxylase (*VpC4H1*) were identified in vanilla pods and studied during maturation. Among phenylpropanoid genes, differential regulation was observed from 3 to 8 months after pollination. *VpPAL1* was gradually up-regulated, reaching the maximum expression level at maturity. In contrast, genes encoding 4HBS, C4H, OMT2 and OMT3 did not show significant increase in expression levels after the fourth month post-pollination. Expression profiling of these key phenylpropanoid genes is also discussed in light of accumulation patterns for key phenolic compounds. Interestingly, *VpPAL1* gene expression was shown to be positively correlated to maturation and vanillin accumulation.

1. Introduction

Phenylpropanoid biosynthesis is among the most important secondary metabolism pathways in higher plants. It gives rise to a large number of metabolites, including hydroxycinnamic acids, monolignols/lignin, coumarins, benzoic acids, stilbenes, anthocyanins and flavonoids (Dixon et al., 2002; Vogt, 2010). The core reactions of phenylpropanoid pathway involve three enzymes, generally represented by several gene orthologs among genomes: phenylalanine ammonia-lyase (PAL; EC 4.3.1.4), cinnamate 4-hydroxylase (C4H; EC 1.14.13.11), and 4-coumarate: coenzyme A ligase (4CL; EC 6.2.1.12). PAL is the first enzyme in the pathway and catalyzes the conversion of L-phenylalanine to *trans*-cinnamic acid. Afterward, C4H, which belongs to the cytochrome P450 superfamily, hydroxylates *t*-cinnamic acid into *para*-coumaric acid, leading to the production of lignin and flavonoids (Dixon et al., 2002; Ehlting et al., 2006). Thus, PAL is at a metabolically important position, linking primary and secondary metabolism.

Phenolic compounds play a crucial role in fruit development, maturity and attractiveness. Fruit quality is indeed often associated

Abbreviations: C4H, cinnamate 4-hydroxylase; HBAc, *p*-hydroxybenzoic acid; HBAlc, *p*-hydroxybenzyl alcohol; HBAld, *p*-hydroxybenzaldehyde; MAP, months after pollination; OMT, *O*-methyltransferases; PAL, phenylalanine ammonia-lyase; PCAld, protocatechualdehyde; Van, vanillin; VanAc, vanillic acid; VanAlc, vanillyl alcohol; 4HBS, 4-hydroxybenzaldehyde synthase.

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with specific anthocyanins and characteristic benzenoid flavor components, both metabolites deriving from L-phenylalanine produced through the phenylpropanoid pathway (Griesser et al., 2008; Kumar and Ellis, 2001). Because of its wide use in food, beverages and cosmetics, vanillin (4-hydroxy-3-methoxy-benzaldehyde) is one of the most popular flavors coming from the phenylpropanoid pathway. This secondary metabolite is synthesized in the "pods" of aromatic *Vanilla* species, particularly in *Vanilla planifolia*. If vanillin is the most abundant compound, various phenolic derivatives and their glycosyl conjugates are also produced in vanilla pods. In fact, the distinctive vanilla flavor is made up of more than 250 compounds (Kanisawa et al., 1994; Walton et al., 2003). At harvest, green vanilla pods are flavorless. During pod development (from 7 to 10 months depending on the vines), flavor precursors mainly accumulate in the form of non-aromatic glucosidic compounds. Their biosynthesis has been extensively studied in the plant and in cell cultures (Tokoro et al., 1990). Nevertheless, the biosynthesis pathway of vanillin remains a controversial issue (Dixon, 2011; Havkin-Frenkel et al., 1999; Walton et al., 2003). Conversely, the enzymatic hydrolysis of glucovanillin to vanillin (i.e. flavor emergence) has been greatly studied (Havkin-Frenkel et al., 1999; Kanisawa et al., 1994; Odoux et al., 2003).

Two main hypotheses have been advanced to explain glucovanillin biosynthesis from *p*-coumaric acid. The first one proposes an oxidative route with the formation of a coenzyme A ester. In this route, the "ferulate pathway", involves hydroxylation and methylation steps before chain shortening. The second hypothesis proposes a non-oxidative route with chain shortening as the first stage, followed by hydroxylation and methylation of the aromatic ring ("benzoate pathway"). Until now, only a few enzyme steps in the phenylpropanoid pathway in vanilla tissue cultures have been explored (Pak et al., 2004; Podstolski et al., 2002). Among them, an unusual 4-hydroxybenzaldehyde synthase (4HBS), a cysteine protease, was partially purified in vitro (Podstolski et al., 2002). This enzyme converts *p*-coumaric acid non-oxidatively to *p*-hydroxybenzaldehyde and may play a role in vanillin biosynthesis. Proteins, *O*-methyltransferases 2 and 3 (OMT2 and OMT3), were also biochemically and genetically characterized in *V. planifolia*. These enzymes seem to be close to caffeic acid *O*-methyltransferases, even if their potential in *in vivo* substrates is not yet clear (Li et al., 2006). According to the authors, these enzymes are likely to be involved in the biosynthesis of phenolic and flavonol components, which are part of the vanilla flavor.

Gene expression studies could also be a further approach to explore variations in the phenylpropanoid pathway in *V. planifolia* pods. Gene expression analysis helped to understand the signaling and metabolic pathways underlying cellular and developmental processes. Of all the methods for quantifying gene expression, reverse transcription quantitative real-time polymerase chain reaction (qRT-PCR) is considered the most reliable, thanks to its superior sensitivity and specificity, even with limited amounts of RNA (Van Guilder et al., 2008). Nevertheless, in order to accurately and reliably quantify gene expression, endogenous reference genes must be selected. Genes encoding transcripts involved in basic cellular metabolism are frequently used for this purpose. The six most frequently used reference genes are *Actin*, *β-tubulin*, *Elongation Factor 1 α* (*EF1*), *18S rRNA*, *Glyceraldehyde-3-Phosphate Dehydrogenase* (*GAPDH*) and *Ubiquitin* (Kumar et al., 2011).

To the best of our knowledge, no research has been carried out to evaluate gene expression over time in *V. planifolia* pods and, specifically, within the phenylpropanoid pathway. Consequently, the purpose of the present work is to identify *PAL* and *C4H* gene orthologs that are actively transcribed in developing pods and then to quantify their expression levels during pod maturation, using qRT-PCR, as well as for the previously characterized *Vp4HBS*, *VpOMT2* and *VpOMT3* genes. Nevertheless, in order to explore changes in gene expression, a necessary prerequisite was to identify reliable reference genes as internal normalization control. In addition, expression profiling of these key phenylpropanoid genes is discussed in light of accumulation patterns for metabolites involved in flavor precursors, i.e. *p*-hydroxybenzoic acid, *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde, protocatechualdehyde, vanillic acid, vanillyl alcohol, and vanillin.

2. Methods

2.1. Plant material

V. planifolia vines were grown in a shade house of the "Coopérative Provanille" (Saint-André, La Réunion). When flowers appeared, they were hand-pollinated and labeled. Harvest dates were defined in accordance with the beginning of vanillin and its glycosyl conjugate (glucovanillin) accumulation in pods (Kanisawa et al., 1994; Havkin-Frenkel et al., 1999). Thus, four pods were collected every month, from 3 to 8 months after pollination (MAP). They were randomly sampled in duplicate from different plants, immediately frozen in liquid nitrogen and stored at −80 °C until utilization. These pods were used for metabolomic analysis (Palama et al., 2009). For the present study, the same pods were used for RNA extraction and gene expression studies.

2.2. RNA extraction and cDNA synthesis

For each stage, frozen pods were ground together to a fine powder in liquid nitrogen with a mortar and a pestle. The pools obtained were then analyzed in duplicate for RNA extraction.

Total RNA was extracted from 100 mg homogenized plant material using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Putative genomic DNA contamination was eliminated by treatment with recombinant DNase I (RNase-Free DNase Set, Qiagen). In order to increase its quality, total RNA was finally purified and concentrated using RNeasy MinElute Cleanup columns (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were estimated by spectrophotometry at 260 and 280 nm. Only RNA samples with 260/280 ratios upper 1.9 were used for cDNA synthesis. RNA integrity was also evaluated by agarose gel electrophoresis.

cDNA was synthesized from 1 μg of total RNA for each sample using the ImProm-II Reverse Transcription System Kit (Promega, Madison, USA) in a final volume of 20 μL, as per the manufacturer's instructions. Two independent reverse transcription reactions were performed for each sample. Reactions without the reverse transcriptase were also performed to check the absence of genomic DNA (No-RT controls). The final cDNA samples were then diluted 40-fold prior to use in qRT-PCR.

2.3. Partial cDNA sequences

At the beginning of this work, only a few sequences for *V. planifolia* and orchids were available in public databases, including *VpOMT2* (GenBank ID: DQ400399), *VpOMT3* (GenBank ID: DQ400400). *Vp4HBS* was also available (Havkin-Frenkel et al., 2003). But for classical housekeeping genes such as *β-tubulin*, *Actin*, *EF1*, and other candidate genes like *VpPAL1* and *VpC4H1*, sequences were not available in libraries. For those genes, degenerate primers were used to obtain specific PCR fragments.

Primers were designed using the CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primers) strategy (http://www.bioinformatics.weizmann.ac.il/blocks/codehop.html).