

## VIEWPOINT

# Is a spice missing from the recipe? The intra-cellular localization of vanillin biosynthesis needs further investigations

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## ABSTRACT

Vanillin is the most popular flavor compound in the world. Substantial effort were made in the last decades to completely elucidate the metabolic pathway that leads to vanillin in plants, with some controversy reported. In *V. planifolia*, vanillin biosynthesis occurs in plastids or in redifferentiated-plastids termed “phenyloplasts”. More recently, it was shown that all enzymes required for the conversion of [<sup>14</sup>C]-phenylalanine to [<sup>14</sup>C]-vanillin-glucoside are confined within that organelle. However, knowing that some of these enzymes are cytosolic or ER-membrane bound in most plant species, it raises questions on the interpretation of data obtained from the technique used and on the true localization of the biosynthetic enzymes in *V. planifolia*. In addition, intense debate has emerged about the real participation of last enzyme of the pathway involving vanillin synthase (VpVAN) in the direct conversion of ferulic acid to vanillin. With the discovery of another enzyme capable of this conversion and the lack of activity of VpVAN *in vitro*, further disagreement emerged. One additional challenge to VpVAN being necessary and sufficient is that the transcript for this protein is abundant in various non-vanillin-producing tissues of the vanilla plant. In this viewpoint, we discuss the findings surrounding the cellular-localization and activity of enzymes of vanillin biosynthesis. This will help to further understand the pathway and urge for additional research study to resolve the debate.

## INTRODUCTION

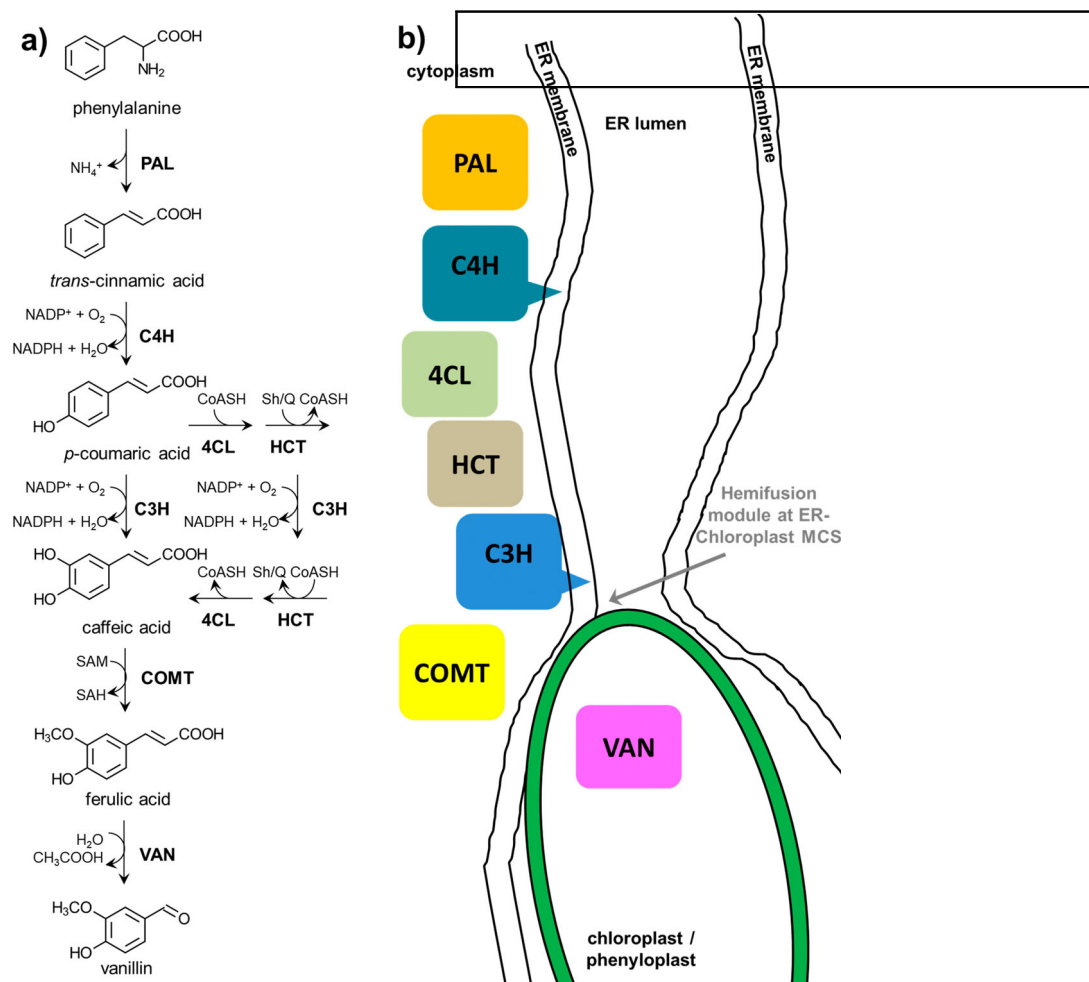
Vanilla extract is a complex mix of over 200 aromatic compounds extracted from cured vanilla pods, from which vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major and most popular flavour compound in the world (Sinha *et al.* 2008). The main natural resource is obtained from the pods obtained by cultivating vanilla orchids, mainly *Vanilla × tahitensis*, *V. pompona* and *V. planifolia*, the last being the most cultivated for commercial production because of its aromatic qualities (Gallage & Møller 2018). Considerable efforts have been made to completely elucidate the vanillin biosynthesis pathway in *V. planifolia*, with some disagreement reported. However, it is widely accepted that L-phenylalanine and hydroxycinnamic acids (cinnamic, *p*-coumaric, caffeic and ferulic acids) are precursors of vanillin biosynthesis (Fig. 1a) (Negishi *et al.* 2009; Gallage *et al.* 2018). However, there is still debate around the identity of the final enzyme and the intracellular localization of the metabolic pathway. Given its commercial importance, vanillin biosynthesis needs to be resolved to provide a promising toolkit that synthetic biologists can use for alternative production.

## VANILLIN BIOSYNTHESIS

Several possible phenylpropanoid metabolic pathways (CoA-dependent or not,  $\beta$ -oxidative or not) leading to vanillin have

been proposed. Pulse-chase experiments with <sup>14</sup>C-labelled compounds showed that the biosynthetic pathway for vanillin is cinnamic acid → 4-coumaric acid → caffeic acid → ferulic acid → vanillin in mature vanilla pods (Negishi *et al.* 2009). In *V. planifolia*, vanillin biosynthesis occurs in plastids or ‘phenyloplasts’, which are differentiated plastids. It was reported that all the enzymes required for the conversion of [<sup>14</sup>C]-phenylalanine to [<sup>14</sup>C]-vanillin glucoside are confined within that organelle (Gallage *et al.* 2018).

Vanillin biosynthesis starts from primary metabolism *via* the amino acid L-phenylalanine (Fig. 1a). It is first converted into cinnamic acid by phenylalanine ammonia lyase (PAL) and then cinnamate 4-hydroxylase (C4H), a cytochrome P450-dependent monooxygenase that catalyses hydroxylation to form *p*-coumaric acid. Next, coumarate 3-hydroxylase (C3H) [also known as *p*-coumaroyl (shikimate/quinate) 3-hydroxylase] hydroxylates free *p*-coumaric acid or *p*-coumaroyl residue at C3 to produce caffeic acid or caffeoyl residue, respectively (Fig. 1a). Recently, a non-membrane bound C3H enzyme (also known as bifunctional cytosolic ascorbate peroxidase) catalysing the direct 3-hydroxylation of *p*-coumaric acid to caffeic acid was characterized from *Brachypodium distachyon* and *A. thaliana* (Barros *et al.* 2019), but as of yet has not been reported in *V. planifolia*. The CoA-dependent non- $\beta$ -oxidative pathway would act through the enzymatic activities of 4-hydroxycinnamoyl-CoA ligase (4CL), hydroxycinnamoyl transferase (HCT) and C3H to yield caffeic



**Fig. 1.** Simplified biosynthetic pathway of vanillin and its potential intra-cellular localization. (a) Enzymes proposed by Gallage *et al.* (2014, 2018) to convert vanillin from L-phenylalanine. (b) Schematic representation of proposed intra-cellular localization of enzymes involved in vanillin biosynthesis. The basis of the diagram showing the continuity of chloroplast and ER membranes used freeze-fracturing techniques, – complementation and regulation of enzyme activities (McLean *et al.* 1988; Mehrshahi *et al.* 2013). Overlapping black and green lines depict the two outer leaflets merging on each side. Full names of compounds are provided in the text.

acid, which is methylated by caffeic acid *O*-methyl transferase (COMT) to produce ferulic acid (Fig. 1a).

A CoA-independent, non- $\beta$ -oxidative pathway has been proposed, where hydroxycinnamic acid intermediates undergo lateral chain shortening *via* a reverse aldol reaction, releasing acetate and benzaldehyde. In *V. planifolia*, a 4-hydroxybenzaldehyde synthase (HBS) has been purified and shown to convert *p*-coumaric acid to 4-hydroxybenzaldehyde and acetate (Podstolski *et al.* 2002).

Gallage *et al.* (2014) identified the last reaction involving the conversion of ferulic acid to vanillin. The enzyme, named vanillin synthase (VpVAN), belongs to the hydratase/lyase type (Gallage *et al.* 2014). Even though a complete biosynthetic pathway has been proposed for vanillin, there is still disagreement around the catalytic activity of VpVAN. Indeed, Yang *et al.* (2017) investigated the enzymatic activity of VpVAN in heterologous organisms (*Escherichia coli*, *Saccharomyces cerevisiae*, *Medicago truncatula* hairy roots, and *Arabidopsis thaliana* seedlings) and repeated the *in vitro* transcription/translation experiment that was first used to characterize

VpVAN, but were unable to confirm the activity of this enzyme (Yang *et al.* 2017). The differences in the results might be explained by the lack of codon optimizations in the systems used and the use of less sensitive analytical equipment, as discussed by Gallage *et al.* (2018). Dixon (2018) suggested that VpVAN may be part of a protein complex in which another enzyme could assist in vanillin biosynthesis. This suggestion would support the hypothesis that VpVAN alone may not be sufficient to catalyse the conversion of ferulic acid into vanillin (Yang *et al.* 2017). In light of the conflicting results, the debate around VpVAN activity remains. In addition, a second enzyme, named phenylpropanoid 2,3-dioxygenase (PPDiox), was reported to catalyse cleavage of the ferulic acid side chain to form vanillin in *V. planifolia* (Negishi & Negishi 2017). Remarkably, it appears that proteins from two different protein families can catalyse the same reaction.

Independent studies using heterologous systems support the ability of VpVAN to convert ferulic acid into vanillin (Havkin-Frenkel *et al.* 2006; Havkin-Frenkel & Podstolski 2007; Chee *et al.* 2017; Arya *et al.* 2022). For example, Chee *et al.* (2017)

expressed *VpVAN* in callus cultures of *Capsicum frutescens* (where vanillin is naturally produced as a precursor of capsaicin biosynthesis) and reported an increase in vanillin accumulation. Furthermore, metabolically engineered rice cell cultures with *VpVAN* have been used as a plant-based alternative to microbial vanillin production systems (Arya *et al.* 2022). In this study, rice calli derived from embryonic rice cells were engineered with a codon-optimized *VpVAN* gene using *Agrobacterium*-mediated transformation. High-performance liquid chromatography identified the biosynthesis of vanillin in transgenic calli lines (Arya *et al.* 2022). Although neither study directly investigates the catalytic activity of *VpVAN*, they serve as proof-of-concept to confirm its involvement in vanillin production.

### THE CHLOROPLAST WAS REPORTED TO BE SUFFICIENT TO CONVERT L-PHENYLALANINE INTO VANILLIN

In vanilla pods, vanillin is stored as vanillin glucoside in phenyloplasts (Brillouet *et al.* 2014) and using immunochemistry and confocal microscopy, the enzyme *VpVAN* was also located in chloroplasts (Gallage *et al.* 2018), thus making the conversion site of vanillin the same as its storage site. Gallage and colleagues investigated whether chloroplasts contain the whole vanillin biosynthesis pathway using a radioactive labelling technique. Intact chloroplasts from *V. planifolia* pods were isolated using Percoll gradients, then incubated with the radiolabelled precursor [ $^{14}\text{C}$ ]-phenylalanine, resulting in [ $^{14}\text{C}$ ]-vanillin glucoside production, which was detected using thin-layer chromatography. They concluded that all enzymes necessary to convert L-phenylalanine into vanillin glucoside were present in the chloroplast. In this investigation, intactness and purity of the isolated chloroplasts were determined with light microscopy; however, this method does not allow detection of co-isolated fragments from other organelles.

It should be noted that few studies have reported presence of enzymes of the phenylpropanoid pathway in chloroplasts, including PAL and C4H (Saunders & McClure 1975), and unidentified enzymes that can convert *p*-coumaric acid into caffeic acid (Satô 1966). It was also reported that the major isoform of PAL extracted from spinach leaves was located outside of the chloroplast, although two other isoforms were present inside the chloroplast (Nishizawa *et al.* 1979). Subsequently, it was proposed that the interaction between the endoplasmic reticulum (ER) and the chloroplast may be necessary for complete biosynthetic machinery (Møller & Laursen 2021). From a review of various publications, we suggest that the above hypothesis should be seriously considered to clarify the intracellular localization of the conversion of L-phenylalanine into vanillin (Fig. 1b).

### CYTOSOLIC- AND ER-ANCHORED VANILLIN BIOSYNTHETIC ENZYMES MAY CO-PURIFY WITH ISOLATED CHLOROPLASTS

Even though some enzymes are considered to be localized in different cellular compartments, they can sometime interact. As such, it was demonstrated that the cytosolic enzyme PAL1, an isoform from *Nicotiana tabacum* involved in lignin biosynthesis, was able to associate with C4H, a membrane enzyme anchored in the ER with its active site located in the cytoplasm,

to form a 'metabolon' (*i.e.* multi-protein complex) (Achnine *et al.* 2004; Bassard *et al.* 2012a; Bassard *et al.* 2012b). The formation of this protein complex was considered to promote a synergy between these two enzymes. This association was studied in microsomes, vesicle-like particles formed during the breakdown of cells and mostly made of ER fragments. PAL was reported as partially associated with microsomes (Rasmussen & Dixon 1999). It was also demonstrated that, in microsome extracts, *p*-coumaric acid was produced from L-phenylalanine more efficiently than from cinnamic acid (Czichi & Kindl 1975, 1977; Rasmussen & Dixon 1999). This implies that in cell lysates, PAL could associate with the ER through its interaction with C4H. This association could provide the catalytic activity necessary to convert L-phenylalanine to *p*-coumaric acid through the first steps of the phenylpropanoid pathway.

Gallage *et al.* (2018) suggested the chloroplast as the unique site of vanillin biosynthesis from L-phenylalanine. However, this conclusion neglects the proximity and near continuity between the ER and plastids, which has been demonstrated through electron microscopy and trans-organelle complementation investigations (McLean *et al.* 1988; Whatley *et al.* 1991; Kaneko & Keegstra 1996; Mehrshahi *et al.* 2013). The literature regarding the membrane contact sites (MCS) between those two organelles has recently been reviewed (Block & Jouhet 2015; Wang & Dehesh 2018; Liu & Li 2019). Indeed, MCS could be important in several metabolic processes. The ER-plastid MCS have been proposed to allow transfer of intermediate metabolites involved in the biosynthesis of specialized metabolites, such as vitamins and lipids (Mehrshahi *et al.* 2013; Negi *et al.* 2018; Michaud & Jouhet 2019). For example, the disruption of tocopherol biosynthesis through mutation of three plastid-localized enzymes was complemented *via* retargeting of these enzymes to the ER (Mehrshahi *et al.* 2013), suggesting that enzymes localized in the ER could have access to the nonpolar metabolite pool of the plastids. Another example involves the lipid metabolism of stomatal guard cells Negi *et al.* (2018). It was demonstrated that, compared to mesophyll cells, the low biosynthesis of lipids in the prokaryotic pathway, which is restricted to the plastids, was compensated by the eukaryotic pathway involving intermediates from the ER. The possible metabolism of lipids from the ER-plastid MCS was reviewed by Michaud & Jouhet (2019). The interaction between the ER and the plastids during biosynthesis of vanillin or other phenylpropanoids has not previously been reported. This could be a topic for further studies on vanillin biosynthesis.

In addition, the strong attraction between the ER and chloroplasts has been demonstrated through optical fragmentation of *A. thaliana* protoplasts (Andersson *et al.* 2007a,b). Protoplasts were fragmented with a nitrogen laser, and a chloroplast captured using optical tweezers. The displacement of the captured chloroplast caused stretching of the ER strands that accompanied the moving plastid (Andersson *et al.* 2007b). Moreover, confocal microscopy observation of isolated chloroplasts showed co-localization between fluorescence from GFP, coming from the ER lumen, and the chloroplasts (Andersson *et al.* 2007a,b). These authors also detected activity from two ER-associated enzymes, NADH-dependent cytochrome c reductase and phosphatidylcholine synthase, in isolated chloroplasts (Andersson *et al.* 2007b), highlighting the possibility that ER fragments, potentially attached through MCS, could be co-isolated with chloroplasts.

Additional investigations based on 'omics' sciences might identify candidate enzymes of the vanillin biosynthetic pathway as well as their subcellular localization. Multiple strategies based on subcellular proteomics have been reviewed recently (Christopher *et al.* 2021) and could be useful for this analysis. A starting point could be identifying candidate enzymes involved in vanillin biosynthesis that are found in isolated chloroplasts, then confirming their localization using immunofluorescence in intact cells. If all the necessary enzymes are detected into the plastids, this would reinforce the conclusion of Gallage *et al.* (2018). If the enzymes proposed by Gallage *et al.* (2018) are located inside the chloroplast, a method could be developed to inactivate enzymes found outside the isolated chloroplast before incubating them with the radiolabelled precursors. Using this method, the precursors will not be consumed by enzymes co-purified with the intact chloroplasts. The impact of contaminating proteins will be the main challenge faced by researchers working on vanillin synthesis.

## CONCLUSION

Vanillin is the most widely used flavour compound in the world, and is employed extensively in food, beverage, perfume and pharmaceutical industries. Several biosynthetic pathways for vanillin have been proposed (Havkin-Frenkel & Belanger 2007; Dixon 2010; Kundu 2017; Khoyraty *et al.* 2018), but a complete CoA-independent non- $\beta$ -oxidative pathway involving VpVAN for the last step was suggested (Gallage *et al.* 2014). Although there is intense debate on VpVAN activity, recent independent studies using heterologous systems support the ability of VpVAN to convert ferulic acid into vanillin. Gallage *et al.* (2018) isolated chloroplasts to describe their biosynthetic capacity to form vanillin. In their study, the method used to isolate chloroplasts did not preclude the co-isolation of fragments from other organelles that could contain vanillin biosynthetic enzymes. The chloroplastic localization of VpVAN has

been demonstrated, but the localization of other enzymes of the vanillin biosynthetic pathway has not (Gallage *et al.* 2018). We propose that cytosolic and ER-anchored enzymes could have been co-isolated with the chloroplasts studied by Gallage and colleagues. Furthermore, it's possible that those extra-chloroplastic enzymes could be implicated or even necessary to complete the conversion of L-phenylalanine into vanillin (Fig. 1b). Based on the diverse investigations presented herein, we suggest that localization of the vanillin biosynthetic machinery needs re-evaluation.

## CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## AUTHOR CONTRIBUTIONS

AD conceived the idea and wrote the first draft of the manuscript. SB and IDP completed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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