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Molecules of Interest

Vanillin

Nicholas J. Walton*, Melinda J. Mayer, Arjan Narbad

Food Safety Science Division, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK

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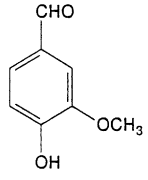
Abstract

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is an important flavour and aroma molecule, but is also of interest because of its biogenetic relationship to the phenylpropanoid pathway and to other molecules of physiological significance, notably salicylate. Recent progress towards characterisation of the biosynthesis of vanillin is reviewed. In *Vanilla*, there is some evidence that the route to vanillin-β-D-glucoside may proceed from 4-coumaric acid via 4-hydroxybenzaldehyde, with glucoside formation occurring not necessarily as the final step, and possibly with the involvement of 4-hydroxybenzyl alcohol β-D-glucoside tartrate bis-esters as "shunt" metabolites. This appears to be given tentative support by the recent partial characterisation of a 4-hydroxybenzaldehyde synthase from *Vanilla*. On the other hand, a well-characterised, CoA-dependent, non-oxidative chain-shortening mechanism to produce vanillin from ferulic acid, occurring as part of a pathway of hydroxycinnamate degradation in *Pseudomonas*, may not be representative of hydroxycinnamate chain-shortening mechanism(s) occurring in *Vanilla* and other plants. Nevertheless, by expression of the *Pseudomonas* enzyme 4-hydroxycinnamoyl-CoA hydratase/lyase (HCHL), attempts have been made to introduce a direct capacity for vanillin formation into model plants by diversion of the phenylpropanoid pathway. The results obtained have emphasised the obstacles to achieving the desired oxidation level (aldehyde) and ring substitution (4-hydroxy-3-methoxyphenyl), even when a substantial metabolic diversion is successfully achieved. Finally, the significance of the latest biosynthetic and biotechnological developments is reviewed briefly in relation to authentication of vanillin.

Keywords: Vanillin; *Vanilla*; Benzaldehydes; Ferulic acid; Phenylpropanoid; Metabolic engineering

1. Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde; Fig. 1) is the major component of natural vanilla, which is one of the most widely used and important flavouring materials worldwide. The source of vanilla is the bean, or pod, of the tropical *Vanilla* orchid (principally *Vanilla planifolia* Andrews, syn. *V. fragrans* (Salisb. Ames)). The Aztecs of Mexico cultivated *Vanilla*, which was then brought to Europe by the Spaniards after 1520 and is now cultivated in a number of tropical countries, the major producers being Mexico, Madagascar, Tahiti and Indonesia. Vanillin in fact occurs in trace amounts in other plants, including commercial products such as tobacco (Makkar and Beeker, 1994); however, the pods of the *Vanilla* orchid still remain the only commercial source of natural vanillin. Although more than 12,000 tons of vanillin are produced each year, less than 1% of this is natural vanillin from *Vanilla*; the remainder is synthesised much more cheaply *via* chemical processes. The value of vanillin extracted from *Vanilla* pods is variously calculated as being between $1200 per kilo and $4000 per kilo, in contrast to the price of synthetic vanillin, <$15 per kilo (Lomascolo et al., 1999; Muheim and Lerch, 1999). Synthetic vanillin is used in both food and non-food applications, in fragrances and as a flavouring in pharmaceutical preparations. Currently, approximately 50% of the worldwide production of synthetic vanillin is used as an intermediate in the



Vanillin

Fig. 1. Vanillin.

Abbreviations: HCHL, 4-Hydroxycinnamoyl-CoA hydratase/lyase

* Corresponding author. Fax: +44-1603-507723.
E-mail address: nicholas.walton@bbsrc.ac.uk (N.J. Walton).

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chemical and pharmaceutical industries for the production of herbicides, antifoaming agents or drugs such as papaverine, L-dopa, L-methyldopa and the antimicrobial agent, trimethoprim (Hocking, 1997). Synthetic vanillin is also used in household products, such as air-fresheners and floor polishes.

In common with many other low-molecular weight phenolic compounds, vanillin displays antioxidant and antimicrobial properties and hence has the potential for use as a food preservative (Burri et al., 1989; Davidson and Naidu, 2000). It is active against both Gram-positive and Gram-negative food-spoilage bacteria and has been shown to be effective against both yeasts and moulds in fruit purées and laboratory growth media (Cerrutti et al., 1997; López-Malo et al., 1998; Fitzgerald et al., 2003). One limitation is the strong flavour of vanillin at the minimal inhibitory concentrations required, but this may be partially overcome by using it in combination with other, synergistic antimicrobials, thus lowering the effective concentrations that are necessary (Gould, 1996). There is some evidence for antimutagenic effects of vanillin, for example in suppressing chromosomal damage induced by methotrexate in the Chinese hamster V79 cell line (Keshava et al., 1998).

Vanillin is of interest to plant scientists for two main reasons. The first concerns the relationship of vanillin with the phenylpropanoid pathway and with the mechanisms of formation of benzoic acids, including 4-hydroxybenzoic acid and the signalling compound, salicylic acid (2-hydroxybenzoic acid). In particular, the mechanisms of chain shortening of putative phenylpropanoid precursors to benzoic acids in plants have remained elusive for decades. The second relates to the commercial importance of vanillin and to the possibilities of producing the compound by biotechnology. On account of the limited supply and high price of natural vanilla and the predominance of chemically synthesised vanillin, there arose in the 1980's and 1990's an incentive to explore and develop biological sources of "natural" vanillin and vanilla-type flavouring that could be marketed as a realistic alternative to the chemically-synthesised substance. Both these aspects are reviewed here, including whether the molecular genetic characterisation of mechanisms of vanillin formation might open up possibilities to introduce new or enhanced biosynthetic capacities in plants.

2. Biosynthesis of natural vanillin

The flowering of plants of *Vanilla planifolia* is not synchronous and commercial plantations practise hand pollination of the flowers. Approximately six to eight months after pollination, the green vanilla beans are harvested. The vanillin in the green beans is present exclusively in conjugated form, principally as the β-D-glucoside, and at this stage the beans display no trace of the characteristic vanilla flavour. This only develops during the fermentation or "curing" process, which can take more than six months to occur. One of the most obvious aspects of curing is that vanillin β-D-glucoside and related β-D-glucosides come into contact with β-D-glucosidases, with the result that free vanillin and related substances (notably 4-hydroxybenzaldehyde) are released (Kanisawa et al., 1994; Ramachandra Rao and Ravishankar, 2000b; Dignum et al., 2001). The vanillin content of cured pods is usually ca. 2–2.5%, and in addition the number of minor components present is around 200.

The appearance of vanillin during curing is in principle simple, unlike the mechanism by which vanillin β-D-glucoside is initially synthesised. Several biosynthetic routes have been proposed, but much uncertainty remains concerning the chain-shortening and other reactions leading from the putative hydroxycinnamic acid precursor to vanillin β-D-glucoside. In an early study, Zenk (1965) reported the results of feeding radioactively-labelled ferulic and vanillic acids and proposed a route by which both vanillin and vanillic acid were derived from ferulic acid. By analogy with fatty acid β-oxidation, a CoA-dependent β-oxidative cleavage of feruloyl-CoA was suggested, leading to the formation of vanilloyl-CoA; this compound would then be reduced to vanillin (presumably in a reaction analogous to that of cinnamoyl-CoA reductase in the pathway to monolignols) or alternatively deacylated to vanillic acid (Fig. 2).

A quite different and more complex route was later tentatively proposed by Funk and Brodelius (1990a,b, 1992), based on the results of feeding radiolabelled compounds to *Vanilla* tissue cultures (Fig. 3). In this proposal, caffeic acid (3,4-dihydroxycinnamic acid) was first methylated at the 4-position to produce *iso*-ferulic acid (3-hydroxy-4-methoxycinnamic acid), which was then further methylated at the 3-position to produce 3,4-dimethoxycinnamic acid; however, this compound was then *demethylated* at the 4-position, prior to a glucosylation step. Side-chain cleavage (by an unspecified mechanism) was proposed to occur at a late stage, to produce vanillic acid (or its β-D-glucoside); and it was suggested that this was then reduced to vanillin. In contrast, studies from other laboratories showed that addition of ferulic acid to callus cultures or tissue cultures resulted in increased levels of vanillin production (Romagnoli and Knorr, 1988, Labuda et al., 1993) suggesting that ferulic acid might indeed be a precursor of vanillin.

Quite separate evidence that vanillin biosynthesis might be more complex than the intuitively attractive pathway proposed by Zenk (1965) came from measurements of the levels of simple phenolic compounds and their glucosides, during the time-course of development

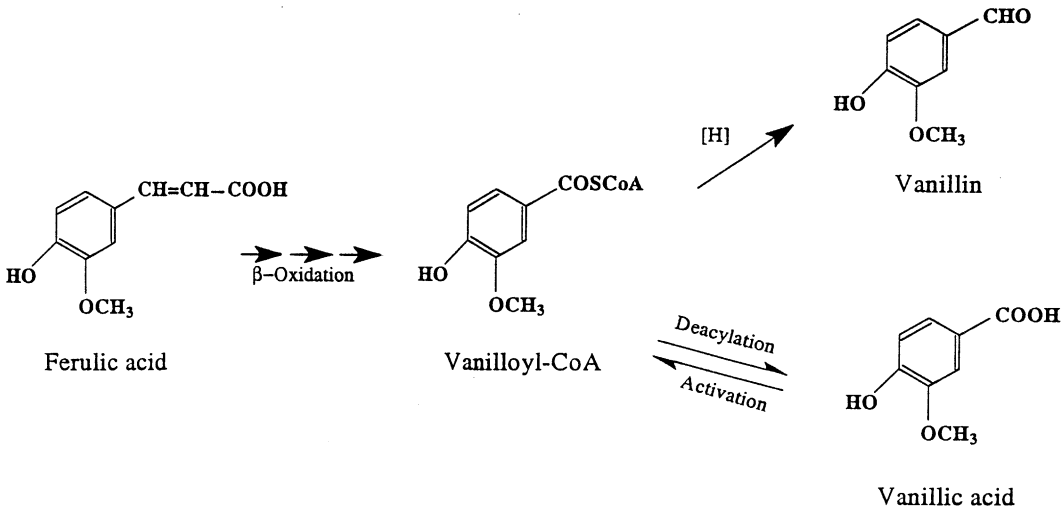

Fig. 2. An outline suggested route of vanillin formation, adapted from Zenk (1965).

of vanilla pods (Kanisawa et al., 1994). In particular, a decline in the level of two major tartrate bis-esters of 4-hydroxybenzylalcohol β-D-glucoside (members of the class of loroglossins found elsewhere in the Orchidaceae) was observed to occur prior to a rise in the level of vanillin β-D-glucoside. A metabolic web involving these tartrate bis-esters as possible "shunt" metabolites was suggested (see Fig. 4).

Aside from work on *Vanilla*, a succession of studies on other plant species has attempted to define the nature of the chain-shortening mechanism by which benzaldehydes and benzoates may be produced from cinnamates. Evidence for the intermediacy of benzaldehydes in the production of benzoates, or for the involvement of CoA in the chain-shortening mechanism, has been inconsistent. French et al. (1976) reported a non-oxidative conversion of 4-coumaric acid to 4-hydroxybenzoic acid in potato tubers. Schnitzler et al. (1992) similarly found this conversion to be non-oxidative and non-CoA-dependent in carrot and to proceed *via* the formation of 4-hydroxybenzaldehyde. In cell cultures of *Lithospermum erythrorhizon*, on the other hand, an initial report (Yazaki et al., 1991) also claimed a non-oxidative, non-CoA-dependent reaction mechanism via 4-hydroxybenzaldehyde as an intermediate, but this was followed by a later publication from the same laboratory describing a CoA-dependent, β-oxidative mechanism that produced 4-hydroxybenzoate without the intermediacy of 4-hydroxybenzaldehyde (Löscher and Heide, 1994). More recently, Jarvis et al. (2000) have reported that benzaldehyde is not an intermediate in the biosynthesis of benzoic acid and salicylic acid in *Cucumis sativus* and *Nicotiana attenuata*. On the other hand, Ahmed et al. (2002) have indicated that benzaldehyde *is* an intermediate in benzoic acid formation in cell cultures of *Hypericum androsaemum* L. (see later).

The reasons behind these different observations, especially in *L. erythrorhizon*, remain unclear, but a new potential model (Fig. 5) for hydroxycinnamate chain-shortening and vanillin formation in plants was revealed with the isolation of 4-hydroxycinnamoyl-CoA hydratase/lyase (HCHL) and its gene from a soil bacterium, *Pseudomonas fluorescens* strain AN103, that had been isolated by growth on ferulic acid as a sole carbon source (Narbad and Gasson, 1998; Gasson et al., 1998; Mitra et al., 1999). This enzyme, a member of the crotonase superfamily, was found to catalyse the hydration and retro-aldol cleavage of 4-coumaroyl-CoA, caffeoyl-CoA and feruloyl-CoA to produce, respectively, 4-hydroxybenzaldehyde, protocatechuic aldehyde and vanillin, together with the other cleavage product, acetyl-CoA. The enzyme showed no detectable activity with cinnamoyl-CoA. The mechanism of the reaction was therefore non-oxidative, yet CoA-requiring—a combination of characteristics not reported in plants. Its catalytic centre activity was found to be low: only 2.3 molecules s^{-1} at 30 °C, with 4-coumaroyl-CoA as substrate (Mitra et al., 1999), and the enzyme accounted for ca. 2–10% of total extractable cellular protein in ferulate-grown cells. The enzyme and gene were demonstrated in other *Pseudomonas* strains (Priefert et al., 1997; Venturi, et al., 1998) and the organisation of the operon was characterised in strain HR199, a strain selected for growth on eugenol (Fig. 5), a major component of clove oil, which is catabolised via ferulic acid (Overhage et al., 1999). An interesting feature from an evolutionary standpoint was the presence of a gene (*aat*) encoding a putative β-ketoacyl-CoA thiolase. It is conceivable that this is a relic of a β-oxidative pathway to vanillic acid, the β-oxidative activity (a 4-hydroxyphenyl-β-hydroxypropionyl-CoA dehydrogenase) having been lost. A

functional homologue of HCHL (62% amino acid identity) is also expressed in the Gram-positive bacterium, *Amycolatopsis* strain HR166, that can produce high yields of vanillin from ferulic acid (Achterholt et al., 2000).

A corresponding activity, cinnamoyl-CoA hydratase/lyase, has now been shown to cleave cinnamoyl-CoA to benzaldehyde and acetyl-CoA in a plant system, cell cultures of *Hypericum androsaemum*, but has not yet been fully characterised (Ahmed et al., 2002). In cell

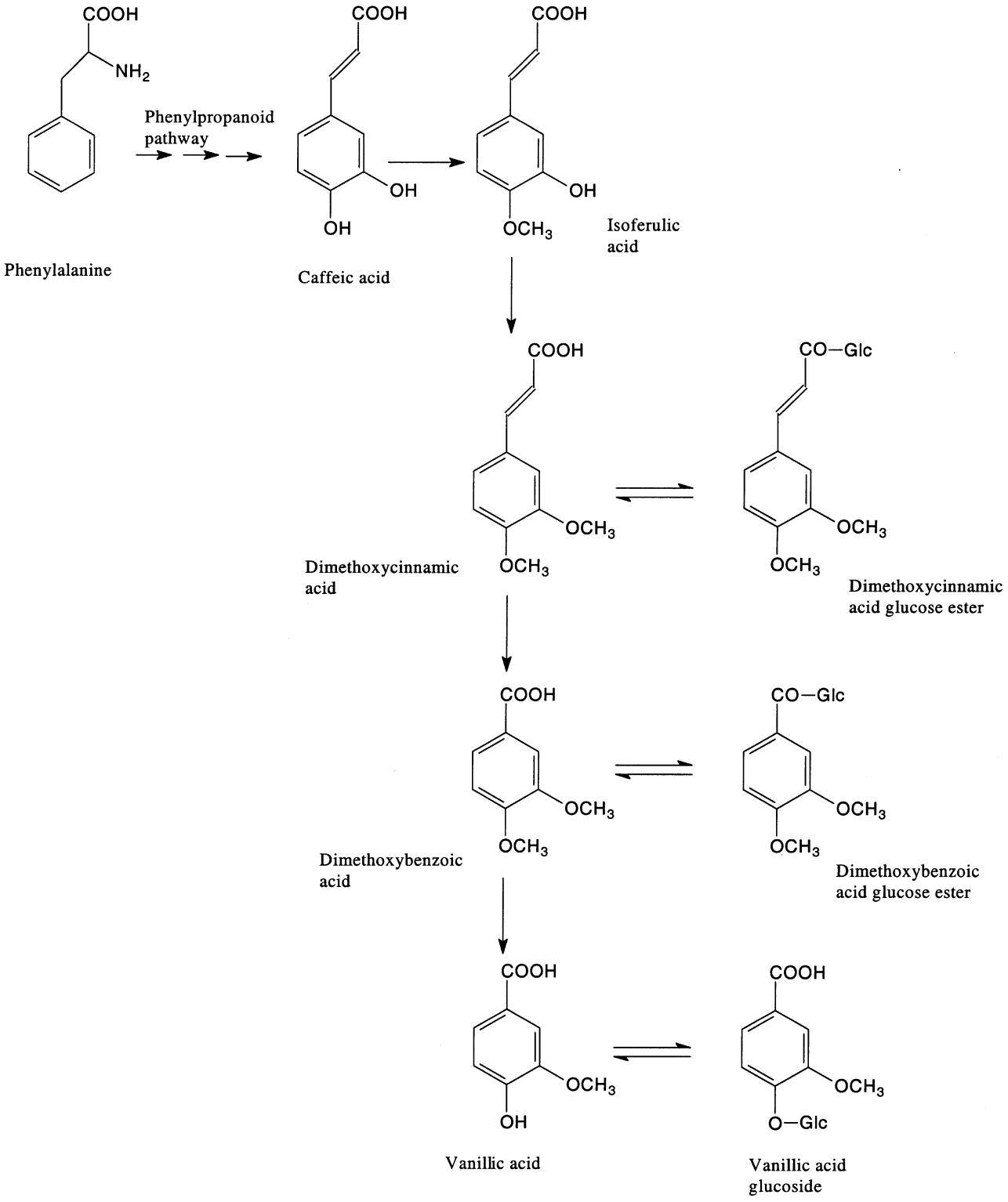

Fig. 3. A route to vanillic acid via isoferulic acid, suggested by Funk and Brodelius (1990a,b, 1992) from work in cell cultures of *Vanilla*.

cultures of *V. planifolia*, by contrast, a partial characterisation has very recently been reported of a quite different hydroxycinnamate chain-shortening activity (Podstolski et al., 2002). The activity, designated 4-hydroxybenzaldehyde synthase, was almost specific for 4-coumaric acid, since cinnamic, caffeic, sinapic and 2-coumaric acids were not accepted as substrates and the activity with ferulic acid was only about 2% of that observed with 4-coumaric acid. The activity was also demonstrated in roots, leaves, pods and embryo cultures of *V. planifolia* and the activity in pods rose substantially at around eight months after pollination, preceding a rise in the 4-hydroxybenzaldehyde content of the pods (determined after hydrolysis with β-glucosidase). The reaction did not proceed via the CoA thioester and although a CoA-dependence could be

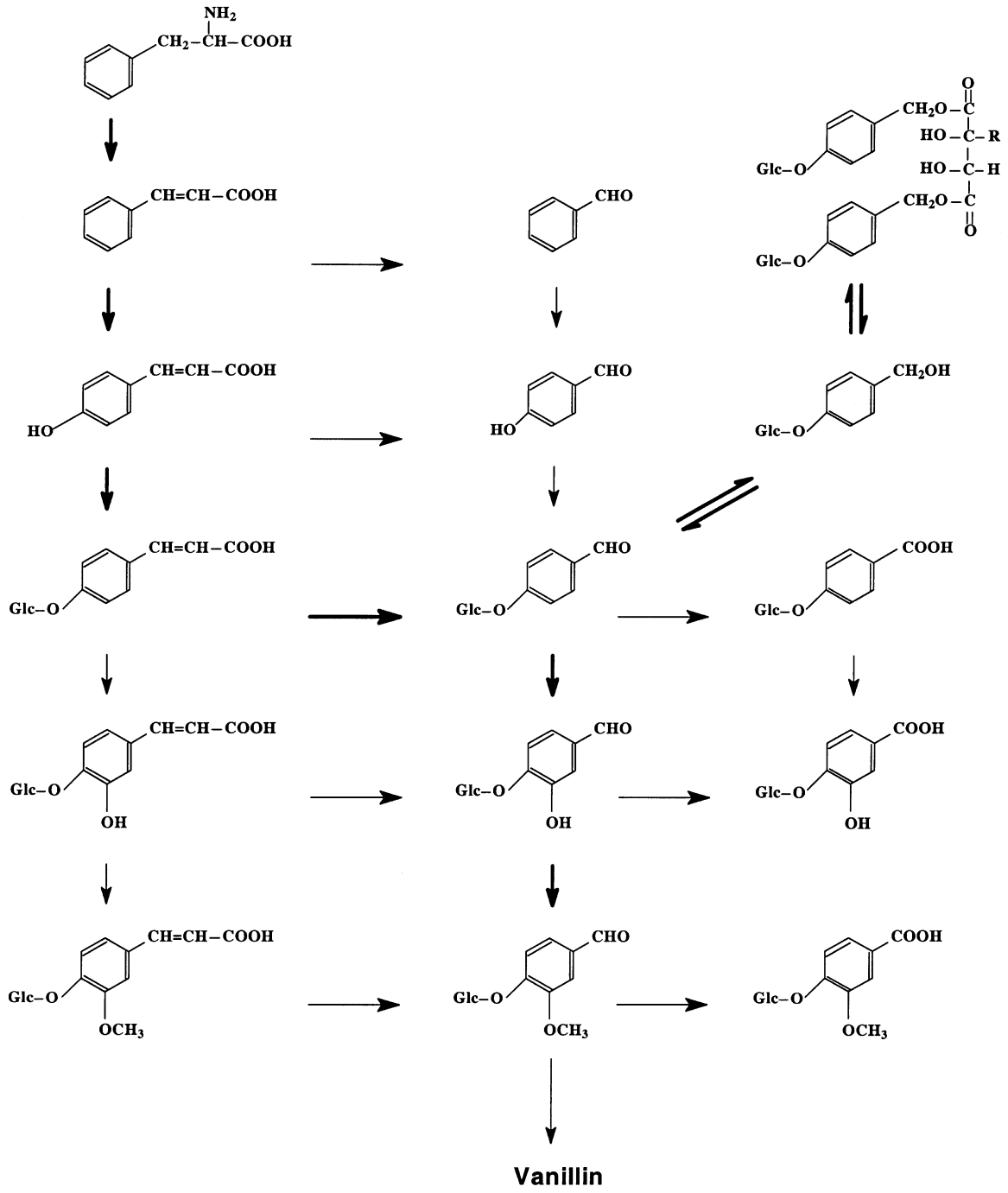

Fig. 4. Suggested biosynthetic relationships of 4-hydroxycinnamic acids, 4-hydroxybenzaldehydes and 4-hydroxybenzoates, and their conjugated derivatives, in developing green vanilla pods (Kanisawa et al., 1994). Two tartrate esters (R = $-CH(CH_3)_2$ and R = $-CH(CH_2CH_3)CH_3$, respectively) were major glucosides in methanol extracts of developing pods.

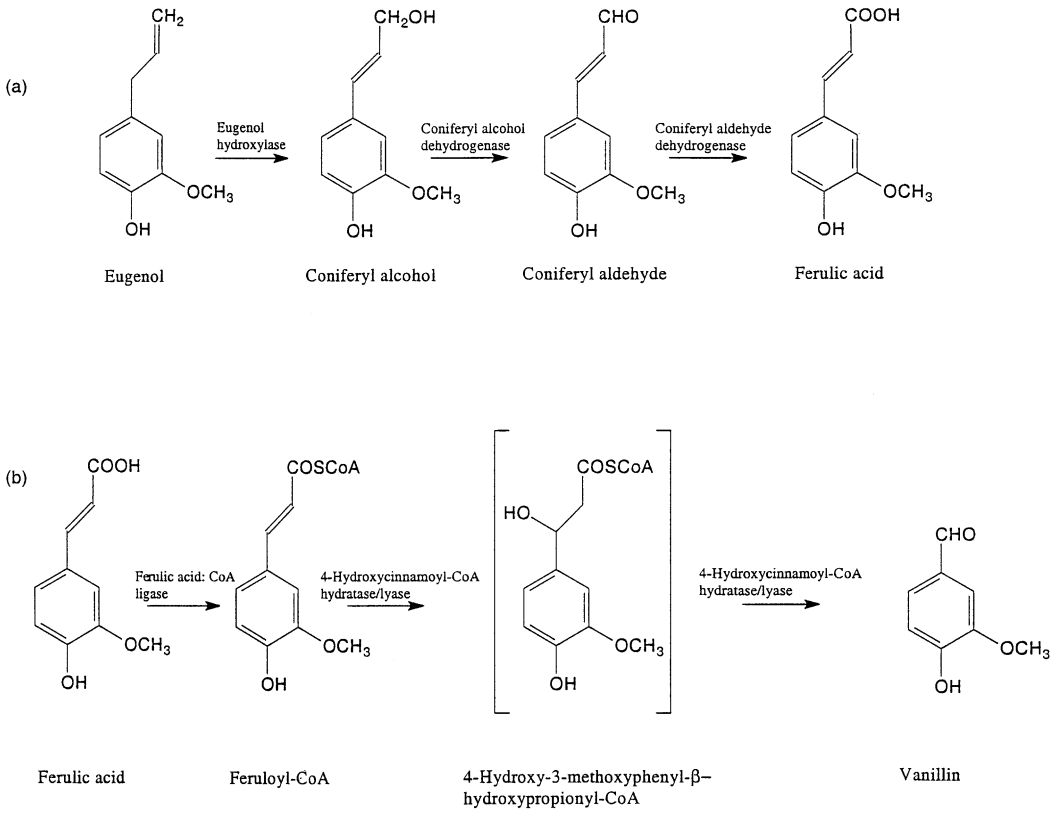

Fig. 5. The route (a) from eugenol to ferulic acid and (b) from ferulic acid to vanillin in *Pseudomonas* strains. 4-Hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA is presumed to be an enzyme-bound intermediate; it is a substrate of HCHL in vitro.

demonstrated, this was non-specific and shown to be due to CoA acting as a thiol reagent since dithiothreitol or dithioerythritol were equally effective. Partial purification of the synthase from *V. planifolia* cell cultures, using ammonium sulphate fractionation, hydrophobic interaction chromatography, ion-exchange chromatography and size-exclusion chromatography, in conjunction with SDS–PAGE analysis, revealed a complex situation with several peaks of activity and a number of protein constituents. Fractions displaying high synthase activity contained a protein of M_r 28 kDa, together with other proteins of M_r 31–45 kDa. Interestingly, the kinetics of the enzyme partially purified using ammonium sulphate fractionation and hydrophobic interaction chromatography revealed a lack of saturation with 4-coumaric acid, even at concentrations as high as 100 mM, together with some indications of positive cooperativity.

The further characterisation of both these enzymes promises to shed valuable light on the diversity of earlier observations. In the meantime, the properties of 4-hydroxybenzaldehyde synthase from *V. planifolia*, and in particular its substrate specificity, would be consistent with chain-shortening as a comparatively early step in the formation of vanillin β-D-glucoside from its phenylpropanoid precursors and with 4-hydroxybenzaldehyde as the initial C_6–C_1 product.

3. Production of vanillin by biotechnology

3.1. Introduction

As indicated at the start of this review, vanillin has been a conspicuous target for biotechnological production by several approaches: the use of enzymes to release or generate vanillin from *Vanilla* and other plant material, the development of tissue cultures, the use of microbial cultures and, finally, genetic modification. This account will be confined to plant-based approaches; vanillin formation using microbial cultures has been reviewed elsewhere (Walton et al., 2000; Priefert et al., 2001).

3.2. Enzymic methods

Several authors have described the use of enzyme preparations containing β-glucosidase to achieve vanillin release from vanilla pods, as an alternative to conventional curing (see Dignum et al., 2001; Ruiz-Terán et al., 2001). Enzymes can also be used in principle to generate vanillin from other plant-derived materials by biotransformation. For example, isorhapontin, a monoglucosylated stilbene constituent of spruce bark, can be cleaved by a dioxygenase isolated from *Pseudomonas* strain TMY1009 (Kamoda et al., 1989). Soybean

lipoxygenase can produce vanillin from esters of coniferyl alcohol (Markus et al., 1992). Van den Heuvel et al. (2001) used a broad-specificity *Penicillium* flavoenzyme, vanillyl alcohol oxidase, to produce vanillin by the biotransformation of vanillylamine (obtainable by the hydrolysis of capsaicin, the main pungent principle of chili peppers) and of creosol (a major component of creosote obtained from heating wood or coal tar). Such approaches are in principle attractive, since the technologies should be reproducible, predictable and acceptable and—given adequate demand, scale-up and stability—the cost of the enzymes may not be prohibitive.

3.3. Cell or organ culture

The possibility of using tissue or organ cultures of *Vanilla* to produce vanillin and related flavour compounds has been explored for some years (Knorr et al., 1993; Ramachandra Rao and Ravishankar, 2000b; Dignum et al., 2001; Priefert et al., 2001). In principle, such cultures might have the potential to produce in addition some of the *ca.* 200 compounds that have been shown to be present in (cured) vanilla pods. *Vanilla* cells and organs and, more recently, cells of *Capsicum frutescens* (Ramachandra Rao and Ravishankar, 2000a), have been cultured successfully and demonstrated to accumulate vanillin and associated metabolites, but production is low.

Low or unreliable levels of production are a generic problem with plant cell cultures, and to a lesser extent with organ cultures (Verpoorte et al., 1999; Walton et al., 1999). The relatively slow growth of plant cell and organ cultures also renders sterility a critical requirement. Commercial production has been feasible in some cases, notably in the case of the red naphthoquinone pigment shikonin produced by cultures of *Lithospermum erythrorhizon*, but in the majority of cases yields are not high enough for processes to be viable economically (Knorr et al., 1993; Verpoorte et al., 1999). A great deal of effort has been expended in empirical approaches to increasing product yields in tissue culture systems, some of which have been applied, singly or in concert, to vanillin production. These have included the feeding of putative precursors (Funk and Brodelius, 1990a,b; Westcott et al., 1994), the use of elicitors or hormones (Funk and Brodelius 1990a, 1992), inhibition of competing pathways (Funk and Brodelius, 1990a), cell immobilisation (Ramachandra Rao and Ravishankar, 2000a; Westcott et al., 1994), adjustment of environmental culture conditions (Havin Frenkel et al., 1996), and the use of an adsorbant, such as charcoal, to sequester the vanillin produced (Westcott et al., 1994). So far, none of these approaches has delivered a commercial cell- or organ-tissue culture system for vanillin production.

3.4. Metabolic engineering for vanillin in plants

Rational approaches to increasing the production of vanillin β-D-glucoside in *Vanilla* are difficult to devise in the absence of an accurate understanding of the biosynthetic route and of the enzymes involved, although the genetic engineering of *V. planifolia* was reported some years ago to be underway (Brodelius and Xue, 1997).

An attractive strategy towards introducing an enhanced, or even completely new, capacity for vanillin formation would be to introduce an enzyme or pathway to generate vanillin from a mainstream intermediate of the plant phenylpropanoid pathway. The isolation of the gene encoding the *bacterial* vanillin-forming enzyme HCHL (see earlier) raised this possibility (Gasson et al., 1998). In principle, feruloyl-CoA, an intermediate of the plant monolignol pathway (Whetten and Sederoff, 1995), could be converted directly to vanillin and acetyl-CoA in a single step. It might be anticipated that any vanillin produced would not be accumulated as the free aldehyde but, in common with the situation in *Vanilla*, would be converted to its β-D-glucoside.

The gene for HCHL was expressed, under the control of a cauliflower mosaic virus promoter with duplicated enhancer sequences, both in plants of *Nicotiana tabacum* (Mayer et al., 2001) and in hairy root cultures of *Datura stramonium* L. (Mitra et al., 2002). In spite of the low catalytic centre activity of HCHL (Mitra et al., 1999), levels of HCHL activity were high in those lines that showed active expression of the gene; in one relatively high-expressing *D. stramonium* root line, HCHL enzyme activity was estimated to be ca. 20% of the endogenous PAL activity. The *N. tabacum* plants actively expressing HCHL showed various pleiotropic effects suggestive of a reduction in carbon flow through the monolignol and flavonoid (including anthocyanin) biosynthetic pathways (see e.g. Elkind et al., 1990), though without definition of the precise mechanism. In particular, the leaf chlorosis and senescence that were observed might have resulted from a decrease in the levels of photoprotective flavonoids, or from impaired xylem function, or from a combination of these or other mechanisms.

Analysis of the soluble phenolic compounds present in *N. tabacum* plants and *D. stramonium* hairy roots expressing HCHL confirmed a major redirection of phenylpropanoid metabolism. Thus, the levels of the major soluble phenylpropanoid-derived compounds normally present in *N. tabacum* leaves, chlorogenic acid and rutin, were very substantially depleted—by as much as 80–90%—in *N. tabacum* lines that were actively expressing HCHL and showing visible phenotypic effects (Mayer et al., 2001). These plants formed new products not detected in control plants: the β-D-glucoside and glucose ester of 4-hydroxybenzoic acid, together

with smaller amounts of vanillic acid β-D-glucoside and of the corresponding β-D-glucoside of 4-hydroxybenzyl alcohol (Mayer et al., 2001). The content of 4-hydroxybenzoic acid β-D-glucoside reached more than 0.2% of the tissue fresh weight in leaves harvested from plants of a high-expressing line at flowering. The highest values for all compounds were found in the seed capsules, which also contained a larger amount, and a higher proportion, of vanillic acid β-D-glucoside. In the *D. stramonium* roots, the picture was somewhat simpler, with two of the compounds, the β-D-glucosides of 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol, comprising the bulk of the new end-products (Mitra et al., 2002). The overall levels accumulated in high-expressing lines were at least comparable with the levels in the *N. tabacum* plants, reaching ca. 0.5% of fresh weight. However, neither in the *N. tabacum* plants nor in the *D. stramonium* hairy roots expressing HCHL was there any evidence of the accumulation of vanillin or 4-hydroxybenzaldehyde or their β-D-glucosides.

The predominance of 4-hydroxyphenyl compounds in these studies suggested that the pool of 4-coumaroyl-CoA was greater than that of feruloyl-CoA and, particularly, of caffeoyl-CoA, since no new 3,4-dihydroxyphenyl compounds were detected at all. 4-Coumaroyl-CoA may itself be a metabolic precursor of caffeoyl-CoA and feruloyl-CoA and therefore the cleavage of 4-coumaroyl-CoA by HCHL could act towards diminishing the pools of both caffeoyl-CoA and feruloyl-CoA. This would be consistent with the pronounced reduction in the levels of chlorogenic acid, a caffeoyl ester, in the *N. tabacum* plants (Mayer et al., 2001). This may not be the whole story, however, since in the *D. stramonium* hairy roots the levels of two putrescine conjugates, caffeoyl putrescine and feruloyl putrescine, were essentially unaffected by the expression of HCHL (Mitra et al., 2002). A number of studies (see Winkel-Shirley, 1999; Rasmussen and Dixon, 1999) have adduced evidence of metabolic channeling in the plant phenylpropanoid pathway. In any event, however, the results suggested that an obstacle to achieving vanillin formation by heterologous expression of HCHL in plants would be the ability to achieve a suitably high feruloyl-CoA/4-coumaroyl-CoA ratio. This was underlined by the fact that the results strongly resembled- in terms of new product formation- those obtained previously by the expression in *Nicotiana* of bacterial chorismate: pyruvate lyase (CPL), an enzyme that can give rise *only* to 4-hydroxybenzoic acid as its reaction product (Siebert et al., 1996; Li et al., 1997).

The apparently complete lack of accumulation of hydroxybenzaldehydes or their glucosides as a result of expression of HCHL (Mayer et al., 2001; Mitra et al., 2002) was probably not a result of toxicity, since vanillin, in common with many compounds, can be glucosylated readily when supplied to cell cultures. For example, cultures of *Catharanthus roseus* accumulated as much as 1.54 g l^{-1} of vanillin β-D-glucoside when supplied with exogenous vanillin (Sommer et al., 1997). It is probable instead that the lack of accumulation reflected high activities of dehydrogenases capable of oxidising the aldehydes, as soon as formed, to the corresponding benzoic acids or, conversely, of reducing them to the benzyl alcohols. Presumably in green vanilla pods these activities are either absent or are compartmented away from their potential substrates.

In summary, what are the prospects for introducing a pathway of vanillin production *de novo* or for enhancing vanillin production in *Vanilla*? At present, HCHL (Gasson et al., 1998; Mitra et al., 1999; Overhage et al., 1999; Achterholt et al., 2000) remains essentially the only enzyme capable of generating vanillin from a phenylpropanoid precursor (feruloyl-CoA) naturally present in plants; 4-hydroxybenzaldehyde synthase from *Vanilla* shows only weak activity with ferulic acid (Podstolski et al., 2002). The key issues would appear to be the supply of free feruloyl-CoA and the activity of enzymes that convert vanillin to vanillic acid and vanillyl alcohol. Addressing the former issue is immediately problematic and could require the up-regulation of both hydroxylase and methylase activities involved in the production of feruloyl-CoA from 4-coumaroyl-CoA via caffeoyl-CoA, or from 4-coumaric acid via caffeic acid and either caffeoyl-CoA or ferulic acid (Whetten and Sederoff, 1995). This is not an attractive option. Alternatively, it is conceivable that a screen of crop plant species and tissues for relative abundances of the hydroxycinnamic acid CoA thioesters might reveal a more suitable target for expression of HCHL. The relatively high levels of vanillic acid β-D-glucoside produced in the capsules of *N. tabacum* plants expressing HCHL suggest this might perhaps be a feasible approach (Mayer et al., 2001). However, preventing the oxidation and reduction of vanillin once formed would not be trivial. It could require the down-regulation of the enzyme activities responsible, which would have to be identified and their genes isolated. It is possible that the enzymes perform other, vital cellular functions and that interfering with these would have deleterious effects. In *Vanilla* itself, the metabolic engineering possibilities are not obvious; there is no reason, for example, to believe that the activity of 4-hydroxybenzaldehyde synthase is limiting to the formation of vanillin β-D-glucoside. In any case, levels of vanillin β-D-glucoside in green vanilla pods are already high and there may not be scope for further enhancement.

4. Determination of the origin of vanillin

Determination of the origin of vanillin is a significant issue commercially since the high price of natural vanilla

from *Vanilla* pods presents an incentive to undertake fraudulent adulteration with synthetic vanillin originating from lignin or guaiacol. This can be detected chromatographically by an abnormal excess of vanillin relative to the profile of minor components in a vanilla preparation, but it is possible to manipulate this profile to some extent artificially.

Looking to the future and considering biotechnological sources of vanillin, one biosynthetic distinction might be based on the phenylpropanoid cleavage mechanism. The enzyme HCHL, found in *Pseudomonas* and *Amycolatopsis* strains, cleaves feruloyl-CoA non-oxidatively, with retention of the proton on the β-carbon as the aldehydic proton of vanillin (Fig. 5). Presumably the non-oxidative 4-hydroxybenzaldehyde synthase of *Vanilla* reported by Podstolski et al. (2002) may behave in this respect similarly. In contrast, the β-oxidative mechanism (Fig. 2) originally suggested by Zenk (1965) (and in addition proposed by Huang et al. (1993) for the conversion of ferulic acid to vanillic acid by *Rhodotorula rubra*) would lead to the loss of this proton.

Biosynthetic origins can be addressed by the analysis of naturally-occurring isotope ratios (in practice, chiefly $^{2}H/^{1}H$ and $^{13}C/^{12}C$), using isotope-ratio mass spectrometry (IR-MS) and nuclear magnetic resonance (Site-specific Natural Isotope Fractionation: SNIF-NMR®). Isotopic ratio characteristics are influenced both by the biosynthetic route itself and, further, by environmental (including climatic) conditions under which biosynthesis occurs (see e.g. Martin et al., 1992; Jamin et al., 1997; Martin, 1998). Thus they can reflect variations in isotope discrimination by individual enzymes operating under differing conditions (e.g. temperature), or between different enzymes, involved for example in alternative biosynthetic pathways to a given end-product, or (most obviously) between an enzymatic and a non-enzymatic synthesis.

Considerable attention has been given to analyses of isotope ratios of vanillin (see Hoffman and Salb, 1979; Krueger and Krueger, 1985; Maubert et al., 1988; Lamprecht et al., 1994; Remaud et al., 1997; Dennis et al., 1998; Martin, 1998; Bensaid et al., 2002). For example, the extent of overall incorporation of naturally occurring ^{13}C, denoted by the $\delta_{PDB}{}^{13}C$ (delta Pee Dee Belemnite) value, for vanillin and 4-hydroxybenzaldehyde samples isolated from *Vanilla* spp. falls within a characteristic range (around −21.0 for vanillin) that reflects the crassulacean acid metabolism (CAM) pathway of photosynthesis by which *Vanilla* fixes CO_2 (Lamprecht et al., 1994; Remaud et al., 1997). This is quite distinct from values determined for samples of vanillin that have been produced from the degradation of lignin, or chemically from fossil fuel sources, where CO_2 is not fixed originally by CAM metabolism (between ca. −25 and −37). Vanillin samples produced by other means, for example by microbial fermentation or by metabolic engineering in plants (or microbes), or enzymically, will display $\delta_{PDB}{}^{13}C$ values that will reflect the mechanism of the pathway involved and the origin of the feedstock (for example, ferulic acid derived from C_3 photosynthesis, as in wheat bran or sugar beet, or derived from C_4 photosynthesis, as in maize). This illustrates the potential of these measurements in the exploration and comparison of metabolic pathways. On the other hand, from an authentication standpoint, the availability of isotopic data for natural and synthetic vanillins provides the means to undertake sophisticated frauds (Krueger and Krueger, 1985; Remaud et al., 1997).

5. Outlook

Vanillin has posed an intriguing biosynthetic problem for many years, closely associated with the more general issue of phenylpropanoid cleavage and the generation of benzaldehydes and benzoates. Within the last few years, encouraging progress has been made in elucidating this process, although a complete biochemical and molecular genetic characterisation in any plant has yet to be achieved. In *Vanilla*, vanillin β-D-glucoside formation appears to be a much more complex process than originally envisaged. Against this background, molecular genetic intervention to introduce or increase vanillin formation in plants is problematic. Furthermore, the commercial incentive may be questionable, given the high yields of vanillin achievable in microbial fermentations (Lomascolo et al., 1999; Muheim and Lerch, 1999; Priefert et al., 2001), the present climate of concern surrounding genetically modified crops and the status of natural vanilla as a high-value product produced by traditional methods.

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