

The Intra-Cellular Localization of the Vanillin Biosynthetic Machinery in Pods of *Vanilla planifolia*

Running head: Vanillin biosynthesis in chloroplasts

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The Intra-Cellular Localization of the Vanillin Biosynthetic Machinery in Pods of *Vanilla planifolia*

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Author contributions

NJG planned and designed the project and performed the [¹⁴C]-radiolabelled precursor feeding experiments, TLC analysis, all the molecular biology analysis, tobacco expression studies, protein extractions, expression studies and contributed to writing the manuscript. **KJ** contributed in planning and carrying out all immunolocalization studies, designing the project and writing the manuscript. **CJ** carried out the DESI imaging analysis. **AJZN** carried out isolation of intact chloroplasts. **TN** performed vanilla/tobacco expression studies. **ED** performed and optimized vanilla crude protein extraction and western blotting analysis. **LD** carried out the vanilla pod cryo-sectioning. **MG** was in charge of vanilla pod sampling and courier shipments of fresh vanilla

materials from La Reunion to Denmark. BLM planned and designed the project, provided biochemical expertise and scientific mentoring and contributed to writing the manuscript.

Abbreviations:

Ampere (A), Carbon dioxide (CO₂), centi (10⁻²) (c), degree Celsius (°C), Dalton (Da), 1,4-dithiothreitol (DTT), endoplasmic reticulum (ER), gravitational acceleration (g), gram (g), hour (h), kilogram (10³g) (Kg), liter (l), mass-to-charge ratio (m/z), meter (m), Metric tons (Mts), minute (min), MicroCurie (μCi), milli (10⁻³) (m), micro (10⁻⁶) (μ), molar (M), phosphate-buffered saline (PBS), potential of Hydrogen (pH) retention time (RT), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), uridine diphosphate (UDP), US Dollar (USD), volt(s) (V), watt(s) (W)

One-sentence summary

Vanillin biosynthesis takes place in the chloroplasts of the *Vanilla planifolia* pod.

Abstract

Vanillin is the most important flavour compound in the vanilla pod. *VpVAN* catalyzes the conversion of ferulic acid and ferulic acid glucoside into vanillin and vanillin glucoside, respectively. Desorption Electrospray Ionization Mass Spectrometry Imaging (DESI-MSI) of vanilla (*Vanilla planifolia*) pod sections demonstrate that vanillin glucoside is preferentially localized within the mesocarp and placental laminae whereas vanillin is preferentially localized within the mesocarp. *VpVAN* is present as the mature form (25 kDa) but dependent on tissue and isolation procedure small amounts of the immature unprocessed form (40 kDa) and putative oligomers (50, 75 and 100 kDa) may be observed by immunoblotting using an antibody specific to the C-terminal sequence of *VpVAN*. The *VpVAN* protein is localized within chloroplasts and re-differentiated chloroplast termed phenyloplasts as monitored during the process of pod development. Isolated chloroplasts were shown to convert [^{14}C]-phenylalanine and [^{14}C]-cinnamic acid into [^{14}C]-vanillin glucoside indicating that the entire vanillin *de novo* biosynthetic machinery converting phenylalanine to vanillin glucoside is present in the chloroplast.

Keywords

Vanillin biosynthesis, *VpVAN*, immunolocalization, chloroplasts, phenyloplasts, vanillin synthase

Introduction

In the last several decades, extensive studies have been carried out to gain a better understanding of the cellular machineries involved in efficient and channelled synthesis of plant natural products. One approach towards elucidation of the biosynthetic pathways involved and the physiological roles of the compounds produced is to determine the tissue, cellular and subcellular localization of their site of biosynthesis and accumulation. In this study, we set out to determine the biosynthetic production site of vanillin (4-hydroxy-3-methoxybenzaldehyde), the world's most popular flavour and aroma compound.

Vanilla and its key flavour component vanillin are universally appreciated flavours. Vanilla is the complete alcohol extract of the mature vanilla seed capsule, commonly called a pod (Sinha et al., 2008). Vanilla plants belong to the *Orchidaceae* family, the genus *Vanilla*, tribe *Vanilleae*, subfamily *Vanilloideae* (Cameron et al., 1999, Cameron, 2004, Cameron and Molina, 2006). Among the approx. 110 vanilla species in the genus *Vanilla*, three species are used for production of vanilla extracts by local farmers as well as at an industrial scale: *Vanilla planifolia*, *V. tahitensis* and *V. pompona*. *V. planifolia* is the most valued among these three species because of its high vanilla flavour quality and, providing 95% of the world vanilla pod production (Odoux and Grisoni, 2010).

V. planifolia is a climbing perennial vine with a large, green and succulent stem that is photosynthetic. The plant produces oblong, smooth, bright green leaves, and adventitious aerial roots that grow opposite each leaf, aiding lateral support. The roots are associated with endotrophic mycorrhiza (Anuradha et al., 2013). The vanilla flowers are yellow, bisexual and develop towards the top of the plant when the vine is approximately four to five meters long. When successful pollination occurs, each flower yields a single pod. The pod reaches its full size about three-months after pollination. The immature green *V. planifolia* pods are almost odourless as the key flavour components are stored as glucosides. Mature pods are approximately 15 cm long and are pale green to yellow in colour (Odoux and Brillouet, 2009). *V. planifolia* pods are harvested when they are eight- to nine-months-old, before the pods begin to dehisce. Freshly harvested pods are processed by curing to stop the natural vegetative processes, to initiate the enzymes responsible for the formation of the aromatic flavour constituents and to prevent microbial growth, thereby enabling long-term preservation (Odoux and Grisoni, 2010).

Figure 1 shows a transverse section of a *V. planifolia* pod to show its anatomy and tissue terminology. The *V. planifolia* pod encompasses three areas, which are visually distinct; the outer part (greener area), inner part (white/yellowish green area) and seeds. The outer part includes epicarp and outer mesocarp. The inner part includes the inner mesocarp, placental laminae, endocarp and seeds. In total, the

mesocarp is formed by 15 to 20 layers of large cells. Seeds are localized towards the cavity of the pod (Fig. 1) (Odoux and Brillouet, 2009, Odoux et al., 2003b).

The vanilla pod is known to produce more than 200 different flavour compounds (Sinha et al., 2008). Vanillin is the most abundant compound and provides the key flavour and aroma of the vanilla extract and of the cured *V. planifolia* pod (Sinha et al., 2008). The compound vanillin is suggested to have various physiological functions in the plant (Burri et al., 1989, Lopezmalo et al., 1995). As vanillin is toxic to living organisms in high concentrations (Boonchird and Flegel, 1982), *V. planifolia* plants store vanillin as vanillin- β -D-glucoside, a conjugated form with glucose, commonly called vanillin glucoside or glucovanillin.

V. planifolia pods are the prime plant organ source of vanillin and the site of vanillin glucoside biosynthesis and storage (Odoux et al., 2003b, Odoux and Brillouet, 2009, Gallage et al., 2014). Vanillin glucoside starts to accumulate in the inner part of the pod when they are three-months-old and continues to do so until the pod is seven- to eight-months-old reaching concentrations above 300 mM in the water phase of the mesocarp cells (Odoux et al., 2003b, Odoux et al., 2006, Odoux and Brillouet, 2009, Palama et al., 2009). Vanillin glucoside was shown by Odoux et al., to accumulate in the inner part of the mesocarp and placental laminae, with the latter two tissues being the main sites (Odoux et al., 2003b). In contrast, Havkin-Frenkel and Dixon reported that vanillin glucoside was produced and accumulated in a unique hairy secretory tissue, the trichomes, and accumulated in the secretion around the seeds (Joel et al., 2003). In a subsequent and thorough study, the conclusions of the latter study were refuted (Odoux and Brillouet, 2009). Vanillin is distributed in similar tissues as vanillin glucoside yet at an about 20 to 50 fold lower concentration (Odoux and Brillouet, 2009, Odoux et al., 2003b). Vanillin and its glucoside are absent from seeds (Odoux and Brillouet, 2009).

Vanillin production in *V. planifolia* may be divided into three modules: synthesis of vanillin via ferulic acid by a C-C chain shortening step, glucosylation of vanillin to vanillin glucoside (the non-toxic storage form) and hydrolysis of vanillin glucoside and liberation of the aromatic compound vanillin.

The biosynthetic pathway of vanillin in the pod of *V. planifolia* has recently been elucidated. Vanillin is synthesized via conversion of ferulic acid and ferulic acid glucoside to vanillin and vanillin glucoside, respectively (Negishi et al., 2009, Gallage et al., 2014). This reaction is catalyzed by a single enzyme, referred to as vanillin synthase (*VpVAN*, accession no. KP278240.1) as demonstrated by the ability of the enzyme to convert ferulic acid/ferulic acid glucoside into vanillin/vanillin glucoside following coupled transcription/translation of the *VpVAN* gene in *in vitro* assays, following transient expression of the gene

in *Nicotiana benthamiana* and following stable expression in *Hordeum vulgare* and *Saccharomyces cerevisiae* (Gallage et al., 2014).

VpVAN has high amino acid sequence similarity to enzymes of the cysteine protease family (Gallage et al., 2014). Cysteine proteases are a large group of enzymes with versatile physiological functions and not very well-defined substrate specificities (Storer and Menard, 1996). Papain (E.C 3.4.22.2) from the latex of *Carica papaya* is the best studied plant cysteine protease (Otto and Schirmeister, 1997, Shindo and Van Der Hoorn, 2008). In general, cysteine proteases are expressed as immature proteins with an N-terminal ER-targeting signal peptide being part of a pro-peptide domain comprising 130-160 residues (Cambra et al., 2012, Wiederanders et al., 2003). In the mature protein, the pro-peptide sequence is removed either by a processing enzyme or by auto-catalytic processing (Turk et al., 2012). Two putative protease cleavage sites in *VpVAN* were predicted at residue 61 (RFAR/RYGK) (Gallage et al., 2014) and residue 135 (VD/GVLPVT) (Yang et al., 2017). The *in vitro* transcription/translation experiments showed no evidence of auto-catalytic processing of the *VpVAN* protein. This indicates that removal of the pro-peptide requires the action of a separate processing enzyme (Gallage et al., 2014). The physiological function of the *VpVAN* pro-peptide is not known. In general, it has been proposed that the pro-peptide sequence may control proper intracellular targeting, may promote proper folding of the mature enzyme and/or serve to maintain the enzyme in an inactive form in the cell to balance its function according to physiological demands (Turk et al., 2012). In the cysteine protease, papain, the presence of the N-terminal pro-peptide blocks the active site cleft directly by non-covalent interactions (Turk et al., 2012). Both the immature as well as mature forms of *VpVAN* were shown to be catalytic active with the latter being more active (Gallage et al., 2014).

The glucosylation step resulting in the conversion of vanillin into its glucoside is much less defined. It is not known at which step glucose incorporation occurs in the biosynthetic pathway. If vanillin glucoside is preferentially made directly from ferulic acid glucoside, the glucosylation may proceed at the level of *p*-coumaric acid, caffeic acid or ferulic acid. In the green pod, vanillin is almost entirely stored as vanillin glucoside demonstrating that the glucosylation of vanillin is highly efficient or alternatively that free vanillin is not involved as an intermediate in vanillin glucoside formation. Our previous proteomic and transcriptomic studies demonstrated that several UDP-glycosyltransferases (UGTs) are present in the *V. planifolia* pod. Of these, *VpUGT72E1* has been shown to be a vanillin specific glycosyltransferase (Gallage et al., 2014).

The third module in vanillin formation involves hydrolysis of vanillin glucoside and liberation of the aromatic compound. Hydrolysis of vanillin glucoside takes place when the cells are broken down as part

of the pod maturation process or as result of pathogen attack and under the curing process. The enzyme which is involved in hydrolysing vanillin glucoside, vanillin β -D-glucosidase, has been well characterized (Odoux et al., 2003a, Odoux et al., 2003b). The β -D-glucosidase was purified to homogeneity and demonstrated to be a tetramer (201 kDa) composed of four identical subunits (50 kDa). The optimum pH was 6.5 and the K_m value for vanillin glucoside was 20.0 mM. V_{max} was 5.0 microkat mg^{-1} (Odoux et al., 2003a). The high K_m value matches the high substrate concentration. At the tissue level, vanillin β -D-glucosidase activity was found to be distributed in the inner mesocarp and placental laminae in a similar manner as vanillin glucoside (Odoux et al., 2003b). The subcellular localization of the β -D-glucosidase throughout pod development was determined using a specific antibody and confocal microscopy. (Brillouet et al., 2014) and demonstrated that the β -D-glucosidase was localized as in a corona around re-differentiating chloroplasts, probably being situated in the lumen between the inner and outer envelopes. In four-months-old pods, few re-differentiating chloroplasts were visible among the photosynthetically active chloroplasts in the cytoplasm of cells of the inner mesocarp. In eight-months-old pods, almost all chloroplasts were re-differentiated as demonstrated by the presence of a corona of β -D-glucosidase and complete loss of chlorophyll red fluorescence. The re-differentiation of chloroplasts was followed by transmission electron microscopy during pod ontogeny and demonstrated a progressive dismantling of the grana thylakoids and thylakoid budding resulting in the formation of circular membrane vesicles packed with ribosomes. At full pod maturity, internal membrane structures were no longer visible but the β -D-glucosidase remained localized as a corona around the re-differentiated chloroplasts (Brillouet et al., 2014).

Brillouet et al, also proceeded to investigate the subcellular localization of vanillin glucoside during pod ontogeny (Brillouet et al., 2014). Using multiple cell imaging approaches combining immunohistochemistry localization by confocal microscopy and fluorescence spectral analysis by multiphotonic microscopy, it was discovered that vanillin glucoside was progressively stockpiled in the inner volume of the re-differentiated chloroplasts as solid amorphous deposits. The vanillin glucoside concentration was estimated to exceed 4 M and the deposition as an amorphous solid was suggested to ensure homeostasis. At the end of the re-differentiation process, the vanillin glucoside deposits filled the entire plastidial volume (Brillouet et al., 2014). Based on the discovered function of the re-differentiated chloroplasts as a storage site for a phenolic plant specialized metabolite, the re-differentiated chloroplasts were named phenyloplasts (Brillouet et al., 2014). In summary, the vanillin glucoside biosynthetic pathway, storage site of vanillin glucoside in re-differentiated chloroplasts termed phenyloplasts as well as the β -D-glucosidase involved in aroma release have been elucidated.

In our current study, we set out to further increase our understanding of how *V. planifolia* orchestrates the biosynthesis of vanillin glucoside in so high concentrations by investigating the cellular and intracellular site of vanillin biosynthesis in the *V. planifolia* pod and the post-translational processing of *VpVAN*. Desorption Electrospray Ionization Mass Spectrometry Imaging (DESI-MSI) was used to visualize the distribution of the main flavour components within the *V. planifolia* pod. A polyclonal antibody specific to the C-terminal sequence of *VpVAN* was used to demonstrate that the *VpVAN* protein and thus vanillin glucoside biosynthesis is localized in chloroplasts and in the re-differentiated chloroplasts termed phenyloplasts. Radio-labelling experiments support that chloroplasts *de novo* synthesize vanillin glucoside from phenylalanine and cinnamic acid.

Results

Vanillin glucoside is localized in the inner part of the vanilla pod where vanillin biosynthetic activity is detected.

Administration of [¹⁴C]-radiolabelled precursors to *V. planifolia* pods harvested at three-, four-, five-, six-, seven-, eight- and nine-months following pollination demonstrated that the pods actively biosynthesized vanillin. The studies with radiolabelled precursors were carried out using *V. planifolia* pods shipped from La Reunion to Copenhagen by courier mail while still attached to their vine. In one set of experiments, [¹⁴C]-phenylalanine, [¹⁴C]-cinnamic acid, *p*-[¹⁴C]-hydroxybenzaldehyde and [¹⁴C]-vanillin were administered to the inner yellow fleshy region of the pod including the inner mesocarp, placental laminae and endocarp. In a second set of parallel experiments, the radiolabelled compounds were administered to the thick dark green outer part of the pod including mainly the epicarp and outer mesocarp. Radiolabelled metabolites formed were separated by thin layer chromatography (TLC) (Suppl. Fig. 1). Upon administration of [¹⁴C]-phenylalanine and [¹⁴C]-cinnamic acid to the inner part of the pod, a radiolabelled product co-migrating with the vanillin glucoside authentic standard was observed while administration of *p*-[¹⁴C]-hydroxybenzaldehyde neither gave rise to formation of radiolabelled vanillin nor vanillin glucoside. These results show that an active vanillin glucoside biosynthetic machinery is situated in the inner part of the *V. planifolia* pod throughout pod development. The time course study also demonstrated that administration of *p*-[¹⁴C]-hydroxybenzaldehyde did not contribute to vanillin biosynthesis at any of the time points examined during *V. planifolia* pod development. It is to be noticed that the administration of radiolabelled [¹⁴C]-vanillin resulted in formation of radiolabelled vanillin glucoside in the inner as well as outer part of the vanilla pod demonstrating that vanillin glucosyltransferase activity was present also in parts of the pod not catalysing *de novo* synthesis of vanillin (Suppl. Fig. 1).

Desorption Electrospray Ionization Mass Spectrometry Imaging (DESI-MSI) was carried out to visualize the localization of the two main metabolites, vanillin and vanillin glucoside, in the *V. planifolia* pod using longitudinal as well as cross sections of a six-months-old *V. planifolia* pod (Fig. 2). In the recordings obtained, the colour intensity of a pixel in each of the compound-specific images are scaled relative to the intensities for that compound in the rest of the image, and not relative to the signal of other detected compounds. MS images thus provide information about the distributions of individual compounds, but not about their mutual abundances. Comparison of absolute intensities obtained with different compounds in the imaging experiment cannot be translated into mutual abundances due to their differences in ionization efficiency.

As shown in Suppl. Fig. 1, radiolabelling experiments based on administration of [¹⁴C]-radiolabelled precursors demonstrated that vanillin biosynthesis takes place in the inner part of the pod. DESI-MS images (Fig. 2, C-D and E-F) visualize the tissue distribution of vanillin and vanillin glucoside, respectively, in a six-months-old pod. Both compounds are present in the tissues where biosynthetic activity was detected. Vanillin glucoside is specifically localized within the inner mesocarp, placental laminae and endocarp (Fig. 2, E-F), while vanillin is distributed within the mesocarp (Fig. 2, C-D). The partly non-superimposed distribution of vanillin and vanillin glucoside suggests that one or more vanillin β -glucosidases and/or vanillin glucosyltransferase activity are differentially distributed at this pod developmental stage (Fig. 2, C-D and E-F).

Presence of VpVAN as demonstrated by Western blot analysis of V. planifolia protein extracts and following transient expression of VpVAN in tobacco leaves

The presence of the *VpVAN* protein was monitored during pod development using a specific *VpVAN* antibody prepared towards the C-terminal peptide (NH₂-) CNWGDNGYFKMELGK (-CONH₂) and used in a 1:5,000 dilution. No background reactions were observed using pre-immune serum from the same rabbit (Suppl. Fig. 2). In agreement with previous results (Gallage et al., 2014), the recognized protein bands at 25 and 40 kDa match the calculated molecular masses of 23.89 and 39.15 kDa of mature and immature *VpVAN*, respectively. These immunoblot patterns demonstrate that *VpVAN* undergoes post-translational maturation in *V. planifolia* as well as in tobacco where the pro-peptide is cleaved off as generally observed for classical cysteine proteases (Gallage et al., 2014, Wiederanders et al., 2003). Comparing the strength of the band patterns, it is apparent that *VpVAN* is present almost entirely in its mature form in seven-months-old *V. planifolia* pods (Fig 3). The highest concentration of vanillin and vanillin glucoside is found in six- to seven-months-old pods. It is thus likely that the activity of the protein is stimulated upon maturation of *VpVAN* (Fig 3).

The formation and post-translational modification of *VpVAN* was further investigated following *Agrobacterium*-mediated transient expression of the *VpVAN* encoding gene in *Nicotiana benthamiana* (tobacco). Crude protein extracts of the *VpVAN*-expressing leaves were obtained seven days after *Agrobacterium* infiltration and probed with the antibody specific to the C-terminal sequence of *VpVAN* at a 1:5,000 dilution (Fig. 3). Western blot analyses showed the presence of a dominant immunoreactive band with an apparent mass of 25 kDa representing mature *VpVAN* indicating effective processing of *VpVAN* into the processed mature form after seven days of transient expression in *N. benthamiana* (Fig. 3).

Mature VpVAN oligomers detected in the crude protein extracts from V. planifolia pods as demonstrated by Western blotting analysis

Cysteine proteinases are known to form dimers (Vincent and Brewin, 2000) and dimerization has been associated with gain of optimal protease activity (Olsen et al., 2009). The possible occurrence of dimers of *VpVAN* was investigated by SDS-PAGE fractionation of a protein extract from a seven-months-old *V. planifolia* pod followed by Western blot analysis using a dilution series of the *VpVAN* C-terminal specific antibody. When the *VpVAN* C-terminal antibody was used at a 1:50 dilution, five protein bands migrating with apparent molecular masses of 25, 40, 50, 75 and 100 kDa were detected (Fig. 4). The *VpVAN* C-terminal antibody did not show cross reactivity towards any of the major protein components in the crude protein extract from *V. planifolia* pod (Fig. 4). In agreement with previous results (Gallage et al., 2014), the 25 and 40 kDa bands match the calculated molecular masses of 23.89 and 39.15 kDa of mature and immature *VpVAN*, respectively and possibly the 50, 75 and 100 kDa bands represent homodimers, trimers and tetramers of the mature *VpVAN*. When the Western blot analyses were carried out using antibodies applied at increasingly higher fold dilutions (Suppl. Fig. 3), the 50, 75 and 100 kDa bands vanished, possibly demonstrating that oligomeric forms of mature *VpVAN* had reduced binding affinity to the antibody compared to the monomer of mature *VpVAN* (Suppl. Fig. 3).

In attempts to convert the putative oligomers of mature *VpVAN* into the 25 kDa monomer, the protein extract from an eight-months-old vanilla pod was reduced with tris(2-carboxyethyl)phosphine (TCEP) before SDS-PAGE. The concentrations of TCEP tested varied from 5 to 50 mM. At none of these concentrations did the presence of TCEP result in reduced band intensities at 50, 75, 100 kDa (Fig. 5 and Suppl. Fig. 4). Oxidation of the protein extract with potassium ferricyanide also did not result in intensification of the 50, 75 and 100 kDa bands (Fig. 5). Based on the specificity of the antibody used we propose that the immunoreactive bands at 50, 75, 100 kDa represent oligomers of the 25 kDa monomer formed as artefacts during homogenization of the vanilla pod. The inner part of the vanilla pod contains

high amounts of oleoresins and a highly viscous mucilaginous material impregnating the proteins upon homogenization of the vanilla pod as previously reported (Odoux, 2005). Random protein cross-linking reactions are thus likely also to result in the formation of oligomers of *VpVAN* not cleavable by TCEP. To reduce protein impregnation with the mucilaginous material and obtain sharper protein bands upon SDS-PAGE, the vanilla pod protein extracts used for Western blots in the current study were extracted in an optimized buffer composed of 10 mM MES (2-(N-morpholino)ethanesulfonic acid), 20mM DTT at pH 5 as recommended for protein extraction of complex fruit tissues (Wang et al., 2008).

VpVAN is localized in the chloroplasts and phenyloplasts as demonstrated by immunohistochemical analyses

The tissue and cellular localization of *VpVAN* in fresh seven-months-old *V. planifolia* pods was examined by confocal microscopy. The immunohistochemical analyses were based on use of the specific antibody to the C-terminal sequence of *VpVAN* and thus targeted both the immature as well as the mature form of *VpVAN*. The images obtained by confocal microscopy clearly demonstrated that the localization of *VpVAN* was restricted to specific plastids present in multiple copies and situated within the cytoplasmic space of the cell (Fig. 6). Control images were obtained using pre-immune serum at the same dilution and in these experiments no reactions mimicking those of the *VpVAN* antibody were observed (Suppl. Fig. 5 and 6). The instrumental gain setting which gave a minimum of fluorescence background when used with the pre-immune serum was also used in the experiments with the *VpVAN* antibody. The fluorescence signal observed using the *VpVAN* antibody thus represents specific immune labelling of *VpVAN*.

Light microscopy of transverse sections of seven-months-old *V. planifolia* pods demonstrated the presence of chloroplasts throughout the entire mesocarp layer (Fig. 7, panels A, D and G). The chloroplasts were likewise detected by their red auto-fluorescence following excitation at 580 nm (Fig. 7, C, F and I). The plastids present in the cytosolic matrix labelled by the specific *VpVAN* antibody were identified as chloroplasts in light microscopy and fluorescence microscopy based studies in which the chloroplasts were identified visually based on their chlorophyll content and red auto-fluorescence in parallel with simultaneous superimposed immunohistochemical localization of *VpVAN* (Fig. 7 A, D and C, F).

It is evident, that some of the antibody-labelled plastids did not show the red auto-fluorescence characteristic of chloroplasts or only weak auto-fluorescence (Fig. 7, B, E and C, F). These plastids have the same overall dimensions as the chloroplasts. Independent of the chlorophyll content and level of red auto-fluorescence, these plastids gave a positive immunochemical reaction with the specific antibody towards *VpVAN*. This indicated that *VpVAN* is localized in chloroplasts as well as in cytoplasmic localized plastids that are devoid of chlorophyll or with reduced chlorophyll content. Re-differentiating

chloroplasts have previously been reported present in vanilla pods and demonstrated to be the main storage sites of vanillin glucoside. The re-differentiated chloroplasts are referred to using the term phenyloplasts and are devoid of chlorophyll (Brillouet et al., 2014). In the merged images (Fig. 7, C and F), the chloroplasts harbouring *VpVAN* are observed as orange (Fig. 7, C and F, white arrows) whereas *VpVAN* containing phenyloplasts completely devoid of chlorophyll are observed as green (Fig. 7, C and F, white stars). With the fluorescence filter settings used to record FITC labelling of the immunolocalized *VpVAN* as well as autofluorescence of chloroplasts, it is very likely that chloroplasts in the transition of being converted into phenyloplasts are observed as having a yellowish colour due to reduced chlorophyll content.

The localization of *VpVAN* was followed at one-month intervals throughout pod development (three- to nine-months). Throughout the entire development phase, *VpVAN* was observed to strictly locate to chloroplasts and phenyloplasts (Suppl. Fig. 7). In fully mature nine-months-old pods, the signals became weak and diffuse (data not shown).

Agrobacterium-mediated transient expression of the gene encoding *VpVAN* in *N. benthamiana* leaves resulted in what would appear to represent a similar sub-cellular localization of *VpVAN* into chloroplasts (Suppl. Fig. 8). However, using this experimental system, strong background fluorescence was observed from the plant cell walls. This strong background fluorescence was also observed in control experiments in the absence of the antibody targeting the C-terminal sequence of *VpVAN*. The use of this analytical system for localization of *VpVAN* was therefore considered not to provide unambiguous conclusions and was not further pursued.

VpVAN detection and functional activity in isolated chloroplasts from V. planifolia

The demonstrated localization of *VpVAN* in chloroplasts, prompted us to isolate intact chloroplasts from eight-months-old *V. planifolia* pods to test their biosynthetic activity. The isolated chloroplast preparations were examined by light microscopy to ascertain their purity and intactness (Fig. 8A). The chloroplasts were isolated following gentle homogenization of the pod tissue in 50mM Hepes (pH 8.0)/0.33M sorbitol and purified using Percoll gradients. Radiolabelled precursors were administered to the isolated intact chloroplasts. Following incubation for 24h, the radiolabelled products formed were separated by thin layer chromatography. The isolated chloroplasts were functionally active as demonstrated by the production of radiolabelled metabolites upon administration of [¹⁴C]-phenylalanine, [¹⁴C]-cinnamic acid and [¹⁴C]-vanillin (Fig. 8). Administration of [¹⁴C]-phenylalanine resulted in formation of [¹⁴C]-cinnamic acid (Fig. 8, 1B). This conversion is catalyzed by phenylalanine ammonia

lyase (PAL) and PAL is known to be localized in the chloroplasts (Sainders and McClure, 1975). [^{14}C]-Vanillin was readily converted to [^{14}C]-vanillin glucoside demonstrating the presence of vanillin glucosyltransferase activity (Fig 8, 3B). Furthermore, a radiolabelled product co-migrating with [^{14}C]-vanillin glucoside was observed upon administration of [^{14}C]-phenylalanine and [^{14}C]-cinnamic acid (Fig. 8, 1B-2B). As expected, strongest labelling of [^{14}C]-vanillin glucoside was observed upon administration of [^{14}C]-cinnamic acid, a more specific vanillin glucoside precursor compared to phenylalanine.

The presence of *VpVAN* in protein extracts obtained from the intact chloroplasts isolated from eight-months-old *V. planifolia* pods were analysed by SDS-PAGE and Western blot experiments (Suppl. Fig. 9-Suppl. Fig. 10). A specific antibody towards photosystem I subunit D (PSI-D) (Haldrup et al., 2003) was used as a positive control (Suppl. Fig. 9). PSI-D is an 18kDa subunit of photosystem I complex and localized on the stromal side of the photosystem I complex in the thylakoid membrane. In most experiments, immunoreactive bands at 25 and 40 kDa corresponding to the mature and immature forms of monomeric *VpVAN* were not observed using the antibody specific to the C-terminal sequence of *VpVAN*. Instead a strong immunoreaction was observed in the 50 kDa region as also observed on the Western blots with crude *V. planifolia* pod protein extracts when *VpVAN* C-terminal antibody was used in dilution 1:300 (Suppl. Fig. 10). Thus, in the isolated chloroplasts, the 25 kDa *VpVAN* monomer was converted into its oligomeric form. Oligomerization may have been favoured by the extended procedure and the high pH of the isolation buffer required for isolation of intact chloroplasts. The isolation of intact chloroplasts from the vanilla pods was hampered by the high amounts of mucilaginous material and oleoresins present in the pods.

Discussion

When the current study was initiated, the enzyme *VpVAN* catalyzing vanillin glucoside synthesis from ferulic acid and the beta glucosidase catalysing liberation of free vanillin had been identified. Likewise, re-differentiated chloroplasts named phenyloplasts had been shown to be the storage site of vanillin glucoside. In the present study, we demonstrate that the chloroplasts and phenyloplasts are also the site of accumulation of *VpVAN*. In agreement with those observations, tracer studies further support that isolated chloroplasts are able to *de novo* synthesize vanillin glucoside.

The *VpVAN* catalyzed synthesis of vanillin glucoside from ferulic acid glucoside proceeds as a retro aldol elimination reaction resulting in C3 side chain shortening of ferulic acid glucoside (Gallage et al., 2014). Such a pathway was initially proposed by Zenk in 1965 and subsequently by Negishi et al. in 2009 based on radioactive precursor studies using [^{14}C]-ferulic acid (Zenk, 1965, Negishi et al., 2009). In the present

study, radiolabelling studies confirmed that active vanillin glucoside biosynthesis takes place throughout pod development (three- to nine-months) and that *p*-hydroxybenzaldehyde did not serve as a vanillin precursor.

DESI-MSI imaging requires minimal sample preparation and thereby minimizes contamination and formation of artefactual compounds. MSI is emerging as an excellent alternative to classical analytical methods where break down of plant constituents may occur in the course of preparation (Bjarnholt et al., 2014). In the present study, DESI-MSI imaging showed localization of vanillin and vanillin glucoside in the inner part of the pod, where vanillin glucoside biosynthetic activity likewise was detected by radioactive precursor administration. The concentration of vanillin is about 20 to 50 fold lower than that of vanillin glucoside (Odoux, 2005) and their distribution patterns are not superimposable. This indicates that β -glucosidase activities involved in vanillin glucoside hydrolysis or glucosyltransferase activities catalysing its formation varies across the placental laminae and mesocarp. A radial distribution of vanillin glucoside and the β -glucosidase enzyme activity has previously been reported (Odoux et al., 2003b). The radiolabelling experiments based on administration of [^{14}C]-vanillin indeed showed the presence of glucosyltransferase activity both in the inner and outer parts of the pod. The localization in the *V. planifolia* pod of vanillin specific glucosyltransferases has not been thoroughly studied.

To determine the subcellular localization of the vanillin biosynthetic machinery, we investigated the cellular and intracellular localization of the key enzyme of vanillin biosynthesis, *VpVAN*. Immunolocalization studies clearly demonstrated that *VpVAN* was localized in chloroplasts distributed within the inner mesocarp and placental laminae of the vanilla pod throughout *V. planifolia* pod development (three- to nine-months). Furthermore, studies of *VpVAN* demonstrated that the protein underwent a maturation step involving removal of the pro-peptide sequence corresponding to mobility shifts on SDS-PAGE from 40 to 25 kDa positions. We previously reported that removal of the pro-peptide sequence augmented the activity of *VpVAN* (Gallage et al 2014). Putative dimers, trimers and tetramers of mature *VpVAN* were observed in crude protein extracts of the vanilla pods and especially in isolated intact chloroplasts. Self-association of proteins forming dimers or oligomers is a common phenomenon. Some cysteine proteases such as caspases are known to undergo dimerization as a requirement to gain catalytic activity and enzyme activation (Grzonka et al., 2001, Marianayagam et al., 2004, MacKenzie and Clark, 2012). In the present study, the oligomeric forms could not be converted into the monomeric form by treatment with a strong reductant like TCEP. Thus, we conclude that these oligomers are artefacts formed as result of a chemical reaction of the *VpVAN* monomer with the abundant mucilaginous material present in the pods.

Administration of [^{14}C]-Phenylalanine to intact chloroplasts isolated from the vanilla pod resulted in formation of a radiolabelled product co-migrating with [^{14}C]-vanillin glucoside suggesting that the entire vanillin biosynthetic machinery responsible for conversion of phenylalanine to vanillin glucoside operated in the chloroplasts. It is not known at which stage in the vanillin pathway the glucosylation takes place. If vanillin glucoside is preferentially made directly from ferulic acid glucoside, the glucosylation may proceed at the level of *p*-coumaric acid, caffeic acid or ferulic acid. The storage form of vanillin in the *V. planifolia* pod is the vanillin- β -D-glucoside. High concentrations of vanillin are toxic to the cell (Boonchird and Flegel, 1982). Accordingly, a vanillin specific UDP-glycosyltransferase would be expected to be co-localized with *VpVAN* to catalyze conversion of any vanillin formed into vanillin- β -D-glucoside to avoid cell toxicity. A study has shown that UDP-glucose, which is the co-substrate for family 1 glucosyltransferases, is *de novo* synthesized in the chloroplasts of *Arabidopsis thaliana* (Okazaki et al., 2009).

The classes of plant natural products that are synthesized in the chloroplasts are remarkably diverse. In addition to photosynthesis, chloroplasts are known to carry out many other essential functions such as synthesis of amino acids (Kirk and Leech, 1972), fatty acids (Lippold et al., 2012), lipids (Wang and Benning, 2012), plant hormones (Metraux, 2002) and vitamins (DellaPenna and Pogson, 2006). Most of these compounds are not only essential for chloroplasts to accomplish their metabolic role but are at different stages of plant ontogeny released from the chloroplast to directly serve as or being metabolized into signalling components for plant growth and development or as defence compounds against pathogens or herbivores (Joyard et al., 2009). Synthesis of aromatic amino acids in the chloroplasts would constantly provide phenylalanine as the initial substrate for vanillin biosynthesis. The phenylalanine precursors, chorismic acid and shikimic acid are known to form esters with *p*-coumaric acid, which are intermediates in ferulic acid biosynthesis. In 1966, conversion of *p*-coumaric acid into caffeic acid was demonstrated in isolated chloroplasts from leaves of *Saxifraga stolonifera*, which shows 4-hydroxycinnamoyl transferase (4-HCL) activity in the chloroplasts (Satô, 1966, Bassard et al., 2012).

While the chloroplast genome encodes about 80–100 proteins, between 2500 and 3500 nuclear-encoded proteins are predicted to be targeted to the chloroplast (Joyard et al., 2009). In general, chloroplast proteins encoded by the nuclear genome and imported into chloroplasts are synthesized as precursor proteins with cleavable N-terminal cTPs that direct each protein to its final destination within the chloroplast sub-compartments (Nielsen et al., 1994, Bruce, 2000). Sequence analysis of the vanillin biosynthetic pathway genes (Gallage et al., 2014) encoding *VpVAN* and putative *V. planifolia* PAL (phenylalanine ammonia lyase), putative *VpC4H* (cinnamate-4-hydroxylase), putative *Vp4HCL* (4-hydroxycinnamoyl transferase), putative *VpHCT* (hydroxycinnamoyl transferase), putative *VpC3H*

(cinnamoyl ester 3' hydroxylase), putative *Vp*COMTs (caffeic acid O-methyl transferase) and putative *Vp*UGT (UDP-glycosyltransferase) using the chloroplast transit peptide prediction program SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) did not provide evidence for the presence of cleavable transit peptides within the encoded proteins. However, the function of chloroplasts as an organelle harbouring entire pathways for secondary metabolites is far from being understood and further biochemical evidence may serve to upgrade the prediction software (Kiessling et al., 2000, McAndrew et al., 2001).

Phenyloplasts have been shown to be the storage site of vanillin glucoside (Brillouet et al., 2014). The phenyloplasts arise following re-differentiation of chloroplasts resulting in loss of their chlorophyll content and photosynthetic abilities and gain of the ability to store high concentrations of phenylpropanoid-derived glucosides (Brillouet et al., 2013, Brillouet et al., 2014). Re-differentiation of chloroplasts to different storage plastids is a well-known phenomenon (Weier, 1936, Leyon, 1953, Kutik, 1998). Leucoplasts are colourless plastids that function as storage organelles. Leucoplasts comprise amyloplasts, oleoplasts and proteinoplasts and these are known to store starch, lipids and proteins, respectively. In fleshy fruits such as tomatoes, ripening is associated with the re-differentiation of green fruit chloroplasts into ripe fruit chromoplasts (Klee and Giovannoni, 2011, Llorente et al., 2016, Llorente et al., 2017). Chromoplasts contain carotenoid pigments that give the red, orange and yellow colours to the plant structure (Roberts, 1946, Egea et al., 2010, Camara et al., 1982, Llorente et al., 2017). Young chromoplasts are metabolically active but contain fewer DNA copies than chloroplasts. Gerontoplasts are known to be the last ontogeny stage of chloroplasts and these plastids no longer harbour functional DNA (Sitte, 1977, Kutik, 1998).

In analogy to the re-differentiation processes of chloroplasts described above resulting in formation of plastids accumulating primary metabolites (amyloplasts, oleoplasts, proteoplasts) as well as secondary metabolite in the form of pigments (chromoplasts) it is not surprising that re-differentiated chloroplasts may also store secondary metabolites as phenylpropanoids. Strong scientific documentation for this was first provided using the vanilla pod as the experimental system. Brillouet et al. coined the term phenyloplasts for this type of re-differentiated chloroplasts (Brillouet et al., 2014). The grana thylakoids of the vanilla chloroplasts were dismantled in the early phases of phenyloplast ontogeny and no deposits of phenolics were observed within the thylakoidal lumen. Based on indirect histochemical data phenyloplasts have earlier been suggested as storage sites for secondary metabolites (Saunders and McClure, 1976, Zaprometov and Nikolaeva, 2003, Liu et al., 2009). Thus, the storage of vanillin glucoside in phenyloplasts may not represent a unique case of sub-cellular sequestration of phenolics in the plant kingdom. Some secondary metabolites accumulate in massive amounts in certain plant tissues. This applies to the cyanogenic glucoside dhurrin which in the tip of etiolated seedlings of *Sorghum bicolor* (L.)

constitutes up to 30% the dry weight (Halkier and Møller, 1989, Saunders and Conn, 1977), and flavan-3-ols which in leaves of *Camellia sinensis* (L.) constitute up to 30% dry weight (Liu et al., 2009). Such high concentrations of secondary metabolites might be attractive to store in phenyloplasts (Gachon et al., 2005).

Biological research concerning the *V. planifolia* orchid, vanilla pods and the physiological role of vanillin formation has gained relatively little attention by the research community in spite of the importance of the vanilla flavour (Gallage and Møller, 2015). Production of vanilla from the vanilla orchid is highly labour intensive and a lengthy process not easily adapted to market demands. Global demand for vanilla was estimated between 2,500 and 3,000 Mts annually in 1998, while the global demand for vanillin was estimated at around 16,000 Mts annually in 2010 and worth USD 650 million in total on the world market (Smolarski, 2012). However, only 0.25% of vanillin originates from cured pods of the vanilla orchid, *V. planifolia* (Gallage and Møller, 2015). Today 99% of all vanillin consumed worldwide is synthetically made, primarily using chemical synthesis based on petrochemicals, or chemically derived by acid hydrolysis of lignin. Market pull and consumer demand have promoted intensive research to develop sustainable biological production platforms for vanillin using microorganisms as a replacement for environmentally unsustainable chemical synthesis (Gallage and Møller, 2015).

The demonstration that vanillin biosynthesis takes place in the chloroplasts would open the door for design of photosynthetic production platforms using algae, cyanobacteria or moss producing vanillin. When fully developed, such production platforms offer superior alternatives to classical biotechnological hosts like bacteria and yeast for vanillin biosynthesis because they use carbon dioxide as sole carbon source and sunlight as the energy source. Production in photosynthetic organisms such as cyanobacteria offers a sustainable alternative, because the carbon skeletons, energy, and reducing power are derived from photosynthesis via CO₂ fixation and light-driven electron transport. Using the cyanogenic glucoside dhurrin as a model system, such photosynthetic systems are now being developed also for synthesis of complex diterpenoids (Møller, 2014, Lassen et al., 2014, Gnanasekaran et al., 2016, Møller, 2017).

In a recent report including Hailian Yang, Daphna Havkin-Frenkel and Richard A. Dixon as authors (Yang et al., 2017), the catalytic activity of *VpVAN* was re-evaluated. It was concluded that the *VpVAN* protein catalyzes conversion of *p*-coumaric acid to *p*-hydroxybenzaldehyde or as an alternative that this conversion proceeds non-enzymatically (Yang et al., 2017). In a previous publication, we studied the catalytic activity of *VpVAN* in *in vitro* and *in vivo* experiments and documented that the *VpVAN* enzyme catalyzes the conversion of ferulic acid and ferulic acid glucoside to vanillin and vanillin glucoside, respectively (Gallage et al., 2014). This result has also been claimed obtained in patent applications (Havkin-Frenkel et al., 2006, Havkin-Frenkel and Podstolski, 2007, Gallage, 2014). The catalytic property

of *VpVAN* as a vanillin synthase has been verified in independent studies demonstrating vanillin and vanillin glucoside formation in *Capsicum frutescens* (hot chili pepper) stably transformed with *VpVAN* (Chee et al., 2017) and further substantiated in the present study.

In the previous studies by Daphna Havkin-Frenkel and Richard A. Dixon, a 28 kDa protein was partially purified from an embryo culture extract of *V. planifolia* and used for preparation of an antibody. As already pointed out by Odoux et al. in 2009, this antibody was not raised towards a single purified protein but based on a protein fraction with the ability to convert *p*-coumaric acid into *p*-hydroxybenzaldehyde. In the recent publication by Yang and co-workers (Yang et al., 2017), the 28 kDa protein reported to be encoded by the same gene sequence as *VpVAN* was isolated from a crude *V. planifolia* embryo culture extract following immune-purification using the antibody described above followed by elution of the protein at pH 2.6. The eluted and supposedly reconstituted protein was reported to convert *p*-coumaric acid to *p*-hydroxybenzaldehyde as monitored by HPLC analysis. No activity was obtained with ferulic acid as a substrate in contrast to what was observed in previous studies (Gallage et al., 2014, Havkin-Frenkel and Podstolski, 2007, Havkin-Frenkel et al., 2006, Chee et al., 2017). In our study we obtained the native immature and mature *VpVAN* protein using a rabbit reticulocyte lysate based transcription/translation system (Gallage et al., 2014). The mature and immature proteins migrated on SDS-PAGE with apparent molecular masses of 25 and 40 kDa, respectively. Using highly sensitive LC-MS (ion trap) analyses, *VpVAN* was demonstrated *in vitro* to catalyze the conversion of ferulic acid into vanillin. No production of *p*-hydroxybenzaldehyde could be detected using *p*-coumaric acid as substrate. These results have been confirmed and further substantiated in the current manuscript.

In our previous publication on *VpVAN* (Gallage et al., 2014), *in vivo* studies were carried out by transient expression of the *VpVAN* encoding gene in *N. benthamiana* and by stable expression of the gene in yeast and barley. In all these studies, a codon optimized gene sequence was used to achieve efficient gene expression. Expression of the transgene was in all cases correlated to formation of vanillin or vanillin derived metabolites whereas formation of *p*-hydroxybenzaldehyde or metabolites thereof was not correlated to transgene expression. The studies reported by Yang and co-workers were carried out without codon optimizations. Heterologous expression of *VpVAN* in host systems such as yeast requires prior modification of endogenous background reactions in the host system to achieve production of vanillin glucoside. Guidance on how to achieve this in combination with sensitive analytics based on use of LC-MS ion trap instrumentation to monitor the activity of *VpVAN* was provided in our previous publication (Gallage et al., 2014).

In conclusion, in this study we demonstrated the tissue and intracellular localization of the vanillin biosynthetic machinery in chloroplasts and re-differentiated chloroplasts termed phenyloplasts during pod development. Vanillin biosynthetic activity was demonstrated in isolated chloroplasts, and *VpVAN* was identified by immunolocalization in both chloroplast and phenyloplast, exactly the plastids that have previously been documented to store vanillin glucoside. Furthermore, *VpVAN* expression studies highlighted that *VpVAN* indeed undergoes a maturation step where the pro-peptide is cleaved off. With these results, we are one step closer in understanding how *V. planifolia* is able to produce vanillin in high concentrations during the five to six months period it develops from an immature to a mature pod.

Materials and Methods

Biological materials

Healthy vines of *V. planifolia* carrying foliage and green vanilla pods were harvested at different time points after pollination and were shipped from the Biological Resource Center (BRC), VATEL, CIRAD, Saint-Pierre, La Réunion, France, by courier carrier to Denmark while maintaining high humidity conditions.

Three-week-old *Nicotiana benthamiana* plants were used for the transient expression of the gene encoding *VpVAN*.

Agrobacterium Strain

Agrobacterium tumefaciens strain AGL1 was used for transient and stable expression assays *in planta* and grown following standard procedures in LB medium with appropriate antibiotics (Bach et al., 2014).

Polyclonal Antibody

A polyclonal antibody was obtained by immunizing rabbits with the *VpVAN* specific peptide sequence (NH₂-)CNWGDNGYFKMELGK(-CONH₂) derived from the C-terminal amino acid sequence of *VpVAN* (amino acids 327-340) (Agrisera AB, Sweden, <http://agrisera.com>). To test the specificity of the antibody, crude protein extracts from seven-months-old pods of *V. planifolia* were separated by SDS-PAGE and subjected to Western blotting.

The specificity of the C-terminal *VpVAN* specific antibody was investigated using a series of antibody dilutions (1:50, 1:100, 1:500 and 1:1,000). When used in a 1:50 dilution, the antibody reacted with proteins migrating with apparent molecular masses of 25, 40, 50 and 100 kDa. In different series of experiments, the crude protein extract was either reduced by incubating with tris(2-carboxyethyl)phosphine (TCEP) (5 to 50 mM, pH 3.5) for 10 min or oxidized by incubating with potassium ferricyanide (10 mM to 50mM, pH 2.95) for 30 min before SDS-PAGE analysis to investigate the presence or formation of protein oligomers. Pre-immune serum from the rabbit used for the antibody production showed no cross-reaction to protein extracts from *V. planifolia* (Suppl. Figure. 2).

Vector constructs

Plant expression vectors for transient expression in tobacco were constructed using Gateway® cloning technology (Life Technologies) as previously described (Gallage et al., 2014).

Biosynthetic assays with green V. planifolia pods and isolated intact chloroplasts

The *V. planifolia* pods harvested at three-, four-, five-, six-, seven-, eight- and nine- months following pollination were cut transverse into 1-2 mm thick discs (approx. 25 mg fresh weight) using a scalpel and further dissected to separate the inner and outer part of the pod. Radiolabelled precursors (0.5 μ Ci) were administered to samples of the inner and outer part of the pod and incubated (30°C) in 400mM Tris/ HCl pH 8, 20mM MgCl₂ for 6-48 h.

The [¹⁴C]-labelled products formed in the experiments with fresh *V. planifolia* pods were extracted by 25% MeOH and applied to Silica Gel 60 F254 TLC plates (Merck, <http://www.merck-chemicals.com>). The plates were developed in ethyl acetate: acetone: dichloromethane: methanol: water (40:30:12:10:8, v/v/v/v/v), dried, exposed (48 h) on phosphor-imaging screens (Molecular Dynamics, <http://www.moleculardynamics.com>) and the radiolabelled products visualized using a Storm 860 Molecular Imager (Molecular Dynamics). Identification of the radiolabelled compounds formed was guided by co-application of authentic standards.

The same experimental procedure was followed using isolated intact chloroplasts (10 μ l, 0.5 μ g chlorophyll/ μ L) obtained from eight-months-old *V. planifolia* pods.

Total protein extraction and Western blot analysis

V. planifolia pods were ground in liquid nitrogen with a mortar and pestle. The resulting powder was homogenized in 100 μ L SDS running buffer (Laemmli buffer, Bio-Rad, US), 300 μ L 10 mM MES buffer (pH 5 to 6) including 20mM DTT. The homogenate was clarified by centrifugation (10,000 x g, 10 min, 4°C). Crude protein extracts were incubated at 65 °C for 30 min before subjected to separation by SDS-PAGE (225V, 30 min, 400mA, 300W) using 12% CriterionTM TGX Stain-Free Precast Gels (Bio-Rad, US) and Precision Plus Protein Stained and Unstained Standards (Bio-Rad, US) as molecular mass markers. Western blots of SDS-PAGE separated proteins were performed using a Trans-Blot R Turbo Transfer Blotting instrument (Bio-Rad, US) and Trans-Blot R Turbo Midi Nitrocellulose membranes (Bio-Rad, US). Membranes were blocked with 5% (w/v) skimmed milk in PBS-T buffer for 2h. The *VpVAN* C-terminal specific antibody was applied in a dilution of 1:5,000. Presence of immunoreactive polypeptides was visualized using a horseradish peroxidase-conjugated goat anti-rabbit antibody (DakoCytomations, <http://www.dako.com/dk>) in a 1:5,000 dilution using the Super Signal West Dura extended duration substrate kit (Pierce, US). In the experiments with pre-sera, the same 1:5,000 dilution of pre-sera and horseradish peroxidase-conjugated goat anti-rabbit antibody were used as advised by the producer (Agrisera).

Blots were developed for 1 to 30 min with a ChemiDoc MP Imaging System (Bio-Rad, US) equipped with a cooled CCD camera (Bio-Rad) set to automatic exposure setting. Total protein was visualized on the membranes using the stain-free blot setting. Precision Plus Protein unstrained ladder (Bio-Rad, US) did not give visible bands below 50kDa, therefore Precision Plus Protein Stained ladder (Bio-Rad, US) was included in the gel. After blotting, the Precision Plus Protein Stained ladder (Bio-Rad, US) can be seen with the naked eye. When imaging with ChemiDoc MP Imaging System, a blot picture was taken before the settings were changed to visualize chemiluminescence. Both blot pictures were then merged to enable visualization of the entire Precision Plus Protein Stained ladder (Bio-Rad, US).

The same procedure was followed to extract total protein and to analyse the presence of *VpVAN* following transient expression of the *VpVAN* gene in tobacco.

Immunolocalization of immature and mature VpVAN in V. planifolia and N. benthamiana

To localize both the immature and mature forms of *VpVAN*, immunolocalization was performed as previously reported (Sanchez-Perez et al., 2012). Infiltrated tobacco leaves or *V. planifolia* pods at different ontogenies were cut into 1cm pieces, embedded in 5% agarose and cut into 120 μm sections. The sections were placed in 5% skimmed milk in PBS at RT to block unspecific background. After 30 min incubation, the C-terminal specific *VpVAN* antibody was added at a 1:100 dilution (Kannangara et al., 2011). Following incubation for 2 h, the sections were washed three times with PBS. Secondary antibody (goat anti-rabbit with FITC fluorophore) was added 1:160 in PBS and incubated for 2 h at RT. Thereafter, the sections were washed three times in PBS and left in PBS.

Sections were mounted with anti-fading agent and analysed by confocal scanning microscopy using Leica SPII at 488 nm excitation for fluorescein isothiocyanate detection (FITC) or with fluorescence microscope, Leica DMR using filters for specific observation of FITC and a combined filter for simultaneous detection of FITC and chloroplast auto-fluorescence.

Chloroplast isolation and fractionation

Intact chloroplasts were isolated from four eight-months-old *V. planifolia* pods essentially as previously reported (Robinson, 2002). Pods were cut transverse into 2 cm long sections and homogenized in HS buffer (50mM Hepes, KOH (pH 8) and 0.33M sorbitol). The homogenate was filtered through nylon mesh (44 micrometer) and centrifuged (3,330 g, 2 min). The chloroplast pellet was gently re-suspended in HS buffer, layered onto pre-cooled Percoll pads and centrifuged (1,400 g, 8 min). The intact chloroplast

sediment was washed in HS buffer, re-sedimented (3,000 g, 2 min) and re-suspended in 50 μ L HS buffer. The purity and integrity of the purified chloroplasts were checked under a Leica ICC50 HD microscope.

Immunoblot analysis of isolated chloroplasts

Samples (40 μ l) were heated at 65 °C for 30 min before separation by SDS-PAGE using 12% Criterion TGX Stain-Free precast gels (Bio-Rad, US) and a Tris/Glycine/SDS running buffer (Bio-Rad, US) at 240 V for 35 min. Proteins were transferred to a 0.2 μ m PVDF membrane using the Trans-Blot Turbo transfer system (Bio-Rad, US) according to the manufacturer's protocol. The membrane was blocked for 20 min at RT with 5% (w/v) skimmed milk in PBS-T buffer and incubated for 1 h with the C-terminal specific *VpVAN* primary antibody at a 1:300 dilution in 2% (w/v) skimmed milk in PBS-T. The blot was washed with PBS-T buffer and incubated with a secondary swine anti-rabbit horseradish HRP-conjugated antibody (Dako) using a 1:5,000 dilution in PBS-T for 1h at RT. The membrane was washed again with PBS-T buffer and the secondary antibody detected using SuperSignal West Dura Chemiluminescent Substrate (Pierce, US) and developed for 1 to 10 min with a ChemiDoc MP Imaging System (Bio-Rad, US). A specific antibody to the photosystem I subunit D (PSI-D) (Haldrup et al., 2003) in 1: 10,000 dilution was used as a reference in quality tests of the isolated chloroplasts.

Desorption Electrospray Ionization Mass Spectrometry Imaging (DESI-MSI)

Pieces of frozen *V. planifolia* pods were mounted on a cryo-microtome sample holder using water as the only adhesive. Using a Leica CM3050S cryo-microtome (Leica Microsystems, Wetzlar, Germany), the tissue was cut into 40 μ m thin sections, which were thaw-mounted on microscope glass slides and stored at -80 °C until the time of analysis. Compared to most other plant tissues, the tissue was very fragile and difficult to section, and therefore a relatively high thickness was chosen. On the day of analysis, the sample slide was taken directly from the freezer to a vacuum desiccator for 10 min prior to DESI analysis. Imaging was performed on a Thermo LTQ XL linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a custom built DESI imaging ion source based on a motorized microscope stage by Märzhäuser Wetzlar (Wetzlar, Germany) and controlled by in-house software. The imaging stage is described in detail elsewhere (Thunig et al., 2011). Imaging was performed in the positive ion mode using a 4 μ L/min flow of methanol and water (80:20) containing 50 mM NaCl and 0.1% formic acid for enhanced generation of sodium adducts and protonated species. The nebulizer gas pressure was 6 bar. The pixel size was 250 μ m. Vanillin was imaged at m/z 153 in its protonated state, vanillin glucoside was imaged at m/z 337 as its sodium adduct, and sucrose (or an isomer) was imaged at m/z 381 as its potassium adduct. The raw data files were converted to imzML files (Schramm et al., 2012), and Data

Cube Explorer (AMOLF, Amsterdam) was used to generate images. MATLAB was used to create coloured overlaid images.

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References:

- ANURADHA, K., SHYAMALA, B. N. & NAIDU, M. M. 2013. Vanilla--its science of cultivation, curing, chemistry, and nutraceutical properties. *Crit Rev Food Sci Nutr.* 53, 1250-76.
- BACH, S. S., BASSARD, J. E., ANDERSEN-RANBERG, J., MOLDRUP, M. E., SIMONSEN, H. T. & HAMBERGER, B. 2014. High-Throughput Testing of Terpenoid Biosynthesis Candidate Genes Using Transient Expression in *Nicotiana benthamiana*. *Methods Mol Biol.* 1153, 245-255.
- BASSARD, J. E., RICHERT, L., GEERINCK, J., RENAULT, H., DUVAL, F., ULLMANN, P., *et al.* 2012. Protein-protein and protein-membrane associations in the lignin pathway. *Plant Cell.* 24, 4465-82.
- BJARNHOLT, N., LI, B., D'ALVISE, J. & JANFELT, C. 2014. Mass spectrometry imaging of plant metabolites--principles and possibilities. *Nat Prod Rep.* 31, 818-37.
- BOONCHIRD, C. & FLEGEL, T. W. 1982. In vitro antifungal activity of eugenol and vanillin against *Candida albicans* and *Cryptococcus neoformans*. *Can J Microbiol.* 28, 1235-41.
- BRILLOUET, J. M., ROMIEU, C., SCHOEFS, B., SOLYMOSI, K., CHEYNIER, V., FULCRAND, H., *et al.* 2013. The tannosome is an organelle forming condensed tannins in the chlorophyllous organs of Tracheophyta. *Ann Bot.* 112, 1003-1014.
- BRILLOUET, J. M., VERDEIL, J. L., ODOUX, E., LARTAUD, M., GRISONI, M. & CONEJERO, G. 2014. Phenol homeostasis is ensured in vanilla fruit by storage under solid form in a new chloroplast-derived organelle, the phenyloplast. *J Exp Biol.* 65, 2427-2435.
- BRUCE, B. D. 2000. Chloroplast transit peptides: structure, function and evolution. *Trends Cell Biol.* 10, 440-447.
- BURRI, J., GRAF, M., LAMBELET, P. & LOLIGER, J. 1989. Vanillin - More Than a Flavoring Agent - a Potent Antioxidant. *J Sci Food Agr.* 48, 49-56.
- CAMARA, B., BARDAT, F. & MONEGER, R. 1982. Sites of Carotenoid Biosynthesis in Pepper (*Capsicum-Annuum-L*) Fruit Chromoplasts. *Cr Acad Sci Iii-Vie.* 294, 649-652.
- CAMBRA, I., HERNANDEZ, D., DIAZ, I. & MARTINEZ, M. 2012. Structural basis for specificity of propeptide-enzyme interaction in barley C1A cysteine peptidases. *PLoS One.* 7, e37234.
- CAMERON, K. M. 2004. Utility of plastid *psaB* gene sequences for investigating intrafamilial relationships within Orchidaceae. *Mol Phylogenet Evol.* 31, 1157-1180.
- CAMERON, K. M., CHASE, M. W., WHITTEN, W. M., KORES, P. J., JARRELL, D. C., ALBERT, V. A., *et al.* 1999. A phylogenetic analysis of the Orchidaceae: evidence from *rbcl* nucleotide sequences. *Am J Bot.* 86, 208-224.
- CAMERON, K. M. & MOLINA, M. C. 2006. Photosystem II gene sequences of *psbB* and *psbC* clarify the phylogenetic position of Vanilla (Vanilloideae, Orchidaceae). *Cladistics.* 22, 239-248.
- CHEE, M. J. Y., LYCETT, G. W., KHOO, T. J. & CHIN, C. F. 2017. Bioengineering of the Plant Culture of *Capsicum frutescens* with Vanillin Synthase Gene for the Production of Vanillin. *Mol Biotechnol* 59, 1-8.
- DELLAPENNA, D. & POGSON, B. J. 2006. Vitamin synthesis in plants: tocopherols and carotenoids. *Annu Rev Plant Biol.* 57, 711-38.

- EGEA, I., BARSAN, C., BIAN, W. P., PURGATTO, E., LATCHE, A., CHERVIN, C., *et al.* 2010. Chromoplast Differentiation: Current Status and Perspectives. *Plant Cell Physiol.* 51, 1601-1611.
- GACHON, C. M., LANGLOIS-MEURINNE, M. & SAINDRENAN, P. 2005. Plant secondary metabolism glycosyltransferases: the emerging functional analysis. *Trends Plant Sci.* 10, 542-9.
- GALLAGE, N. J., HANSEN, E. H., KANNANGARA, R., OLSEN, C. E., MOTAWIA, M. S., JØRGENSEN, K., *et al.* 2014. Vanillin formation from ferulic acid in *Vanilla planifolia* is catalysed by a single enzyme. *Nat Commun.* 5.
- GALLAGE, N. J. & MØLLER, B. L. 2015. Vanillin–Bioconversion and Bioengineering of the Most Popular Plant Flavor and Its De Novo Biosynthesis in the Vanilla Orchid. *Mol Plant.* 8, 40-57.
- GALLAGE, N. J., MØLLER, B. L., HANSEN, E. H., HANSEN, J. 2014. *Vanillin synthase*. PCT/DK2013/050357.
- GNANASEKARAN, T., KARCHER, D., NIELSEN, A. Z., MARTENS, H. J., RUF, S., KROOP, X., *et al.* 2016. Transfer of the cytochrome P450-dependent dhurrin pathway from *Sorghum bicolor* into *Nicotiana tabacum* chloroplasts for light-driven synthesis. *J Exp Bot* 67, 2495-2506.
- GRZONKA, Z., JANKOWSKA, E., KASPRZYKOWSKI, F., KASPRZYKOWSKA, R., LAMKIEWICZ, L., WICZK, W., *et al.* 2001. Structural studies of cysteine proteases and their inhibitors. *Acta Biochim. Pol.* 48, 1-20.
- HALDRUP, A., LUNDE, C. & SCHELLER, H. V. 2003. Arabidopsis thaliana plants lacking the PSI-D subunit of photosystem I suffer severe photoinhibition, have unstable photosystem I complexes, and altered redox homeostasis in the chloroplast stroma. *J Biol Chem.* 278, 33276-83.
- HALKIER, B. A. & MØLLER, B. L. 1989. Biosynthesis of the Cyanogenic Glucoside Dhurrin in Seedlings of *Sorghum bicolor* (L.) Moench and Partial Purification of the Enzyme System Involved. *Plant Physiol.* 90, 1552-9.
- HAVKIN-FRENKEL, D. & PODSTOLSKI, A. 2007. *Vanillin production*. USA patent application, US20070231864 A1.
- HAVKIN-FRENKEL, D., ZYLSTRA, G., FRENKEL, C. & BELANGER, F. 2006. *Production of vanillin in microbial cells*. USA patent application, US 10/532,464.
- JOEL, D. M., FRENCH, J. C., GRAFT, N., KOURTEVA, G., DIXON, R. A. & HAVKIN-FRENKEL, D. 2003. A hairy tissue produces vanillin. *Isr. J. Plant Sci.*, 51, 157-159.
- JOYARD, J., FERRO, M., MASSELON, C., SEIGNEURIN-BERNY, D., SALVI, D., GARIN, J., *et al.* 2009. Chloroplast Proteomics and the Compartmentation of Plastidial Isoprenoid Biosynthetic Pathways. *Mol Plant.* 2, 1154-1180.
- KANNANGARA, R., MOTAWIA, M. S., HANSEN, N. K., PAQUETTE, S. M., OLSEN, C. E., MØLLER, B. L., *et al.* 2011. Characterization and expression profile of two UDP-glucosyltransferases, UGT85K4 and UGT85K5, catalyzing the last step in cyanogenic glucoside biosynthesis in cassava. *Plant J.* 68, 287-301.
- KIESSLING, J., KRUSE, S., RENSING, S. A., HARTER, K., DECKER, E. L. & RESKI, R. 2000. Visualization of a cytoskeleton-like FtsZ network in chloroplasts. *J Cell Biol.* 151, 945-950.
- KIRK, P. R. & LEECH, R. M. 1972. Amino Acid Biosynthesis by Isolated Chloroplasts during Photosynthesis. *Plant Physiol.* 50, 228-34.

- KLEE, H. J. & GIOVANNONI, J. J. 2011. Genetics and control of tomato fruit ripening and quality attributes. *Annu Rev Genet.* 45, 41-59.
- KUTIK, J. 1998. The development of chloroplast structure during leaf ontogeny. *Photosynthetica.* 35, 481-505.
- LASSEN, L. M., NIELSEN, A. Z., ZIERSEN, B., GNANASEKARAN, T., MOLLER, B. L. & JENSEN, P. E. 2014. Redirecting Photosynthetic Electron Flow into Light-Driven Synthesis of Alternative Products Including High-Value Bioactive Natural Compounds. *ACS Synth Biol.* 3, 1-12.
- LEYON, H. 1953. The Structure of Chloroplasts .2. The 1st Differentiation of the Chloroplast Structure in Vallota and Taraxacum Studied by Means of Electron Microscopy. *Exp Cell Res.* 5, 520-529.
- LIPPOLD, F., VOM DORP, K., ABRAHAM, M., HOLZL, G., WEWER, V., YILMAZ, J. L., *et al.* 2012. Fatty acid phytyl ester synthesis in chloroplasts of Arabidopsis. *Plant Cell.* 24, 2001-14.
- LIU, Y., GAO, L., XIA, T. & ZHAO, L. 2009. Investigation of the site-specific accumulation of catechins in the tea plant (*Camellia sinensis* (L.) O. Kuntze) via vanillin-HCl staining. *J Agric Food Chem.* 57, 10371-6.
- LLORENTE, B., D'ANDREA, L. & RODRIGUEZ-CONCEPCION, M. 2016. Evolutionary Recycling of Light Signaling Components in Fleshy Fruits: New Insights on the Role of Pigments to Monitor Ripening. *Front Plant Sci.* 7, 263.
- LLORENTE, B., MARTINEZ-GARCIA, J. F., STANGE, C. & RODRIGUEZ-CONCEPCION, M. 2017. Illuminating colors: regulation of carotenoid biosynthesis and accumulation by light. *Curr Opin Plant Biol.* 37, 49-55.
- LOPEZMALO, A., ALZAMORA, S. M. & ARGAIZ, A. 1995. Effect of Natural Vanillin on Germination Time and Radial Growth of Molds in Fruit-Based Agar Systems. *Food Microbiol.* 12, 213-219.
- MACKENZIE, S. H. & CLARK, A. C. 2012. Death by caspase dimerization. *Adv Exp Med Biol.* 747, 55-73.
- MARIANAYAGAM, N. J., SUNDE, M. & MATTHEWS, J. M. 2004. The power of two: protein dimerization in biology. *Trends Biochem Sci.* 29, 618-25.
- MCANDREW, R. S., FROELICH, J. E., VITHA, S., STOKES, K. D. & OSTERYOUNG, K. W. 2001. Colocalization of Plastid Division Proteins in the Chloroplast Stromal Compartment Establishes a New Functional Relationship between FtsZ1 and FtsZ2 in Higher Plants. *Plant Physiol.* 127, 1656-1666.
- METRAUX, J. P. 2002. Recent breakthroughs in the study of salicylic acid biosynthesis. *Trends Plant Sci.* 7, 332-334.
- MØLLER, B. L. 2014. Disruptive innovation: channeling photosynthetic electron flow into light-driven synthesis of high-value products. In: RYADNOV, B., SUGA (ed.) *Synthetic Biology*. The Royal Society of Chemistry.
- MØLLER, B. L. 2017. Solar-Driven Synthesis of Bioactive Natural Products. *Chem Eng Prog.* 113, 46-54.
- NEGISHI, O., SUGIURA, K. & NEGISHI, Y. 2009. Biosynthesis of vanillin via ferulic acid in *Vanilla planifolia*. *J Agric Food Chem.* 57, 9956-61.
- NIELSEN, V. S., MANT, A., KNOETZEL, J., MOLLER, B. L. & ROBINSON, C. 1994. Import of Barley Photosystem-I Subunit-N into the Thylakoid Lumen Is Mediated by a Bipartite Presequence Lacking an Intermediate Processing Site - Role of the Delta-Ph in Translocation across the Thylakoid Membrane. *J Biol Chem.* 269, 3762-3766.

- ODOUX, E. 2005. Glucosylated aroma precursors and glucosidase(s) in vanilla bean (*Vanilla planifolia* G. Jackson). *FRUITS*. 61, 171-184.
- ODOUX, E. & BRILLOUET, J. M. 2009. Anatomy, histochemistry and biochemistry of glucovanillin, oleoresin and mucilage accumulation sites in green mature vanilla pod (*Vanilla planifolia*; Orchidaceae): a comprehensive and critical reexamination. *FRUITS*. 64, 221-241.
- ODOUX, E., CHAUWIN, A. & BRILLOUET, J. M. 2003a. Purification and characterization of vanilla bean (*Vanilla planifolia* Andrews) beta-D-glucosidase. *J Agric Food Chem* 51, 3168-3173.
- ODOUX, E., ESCOUTE, J. & VERDEIL, J. L. 2006. The relation between glucovanillin, beta-D-glucosidase activity and cellular compartmentation during the senescence, freezing and traditional curing of vanilla beans. *Ann Appl Biol*. 149, 43-52.
- ODOUX, E., ESCOUTE, J., VERDIEL, J. L. & BRILLOUET, J. M. 2003b. Localization of beta-D-glucosidase activity and glucovanillin in vanilla bean (*Vanilla planifolia* Andrews). *Ann Bot*. 92, 437-444.
- ODOUX, E. & GRISONI, M. 2010. Vanilla. In: M, O. E. A. G. (ed.) *Medicinal and aromatic plants*. CRC Press.
- OKAZAKI, Y., SHIMOJIMA, M., SAWADA, Y., TOYOOKA, K., NARISAWA, T., MOCHIDA, K., *et al.* 2009. A Chloroplastic UDP-Glucose Pyrophosphorylase from *Arabidopsis* Is the Committed Enzyme for the First Step of Sulfolipid Biosynthesis. *Plant Cell*. 21, 892-909.
- OLSEN, J. G., DAGIL, R., NICLASEN, L. M., SORENSEN, O. E. & KRAGELUND, B. B. 2009. Structure of the Mature Streptococcal Cysteine Protease Exotoxin mSpeB in Its Active Dimeric Form. *J Mol Biol*. 393, 693-703.
- OTTO, H. H. & SCHIRMEISTER, T. 1997. Cysteine proteases and their inhibitors. *Chem Rev*. 97, 133-171.
- PALAMA, T. L., KHATIB, A., CHOI, Y. H., PAYET, B., FOCK, I., VERPOORTE, R., *et al.* 2009. Metabolic Changes in Different Developmental Stages of *Vanilla planifolia* Pods. *J Agric Food Chem* 57, 7651-7658.
- ROBERTS, E. A. 1946. Electron Microscope Studies of the Structure of Chloroplasts, Chromoplasts, Leucoplasts Showing Their Relationship to Cellulose Particles, Carotin Crystals and Colloidal Carbon. *J Appl Phys.*, 17, 68-68.
- ROBINSON, C., AND MANT, A. 2002. *Molecular Plant Biology* Oxford University Press.2.
- SAINDEERS, J. A. & MCCLURE, J. W. 1975. Phytochrome controlled phenylalanine ammonia lyase in *Hordeum vulgare* plastids. *Phytochemistry*. 14, 1285-1289.
- SANCHEZ-PEREZ, R., BELMONTE, F. S., BORCH, J., DICENTA, F., MOLLER, B. L. & JORGENSEN, K. 2012. Prunasin hydrolases during fruit development in sweet and bitter almonds. *Plant Physiol*. 158, 1916-32.
- SATÔ, M. 1966. Metabolism of phenolic substances by the chloroplasts-II. : Conversion by the isolated chloroplasts of p-coumaric acid to caffeic acid. *Phytochemistry*. 5, 385-389.
- SAUNDERS, J. A. & CONN, E. E. 1977. Subcellular localization of the cyanogenic glucoside of sorghum by autoradiography. *Plant Physiol*. 59, 647-52.
- SAUNDERS, J. A. & MCCLURE, J. W. 1976. The distribution of flavonoids in chloroplasts of twenty five species of vascular plants. *Phytochemistry*. 15, 809-810.
- SCHRAMM, T., HESTER, A., KLINKERT, I., BOTH, J. P., HEEREN, R. M., BRUNELLE, A., *et al.* 2012. imzML--a common data format for the flexible exchange and processing of mass spectrometry imaging data. *J Proteomics*. 75, 5106-10.

- SHINDO, T. & VAN DER HOORN, R. A. L. 2008. Papain-like cysteine proteases: key players at molecular battlefields employed by both plants and their invaders. *Mol. Plant Pathol.*, 9, 119-125.
- SINHA, A. K., SHARMA, U. K. & SHARMA, N. 2008. A comprehensive review on vanilla flavor: Extraction, isolation and quantification of vanillin and others constituents. *Int J Food Sci Nutr.* 59, 299-326.
- SITTE, P. 1977. Chromoplasten — bunte Objekte der modernen Zellbiologie. *Biologie in unserer Zeit.* Wiley Subscription Services, Inc., A Wiley Company.
- SMOLARSKI, N. 2012. *High-Value Opportunities for Lignin: Unlocking its Potential* [Online]. Frost & Sullivan Available: <http://www.greenmaterials.fr/wp-content/uploads/2013/01/High-value-Opportunities-for-Lignin-Unlocking-its-Potential-Market-Insights.pdf>.
- STORER, A. C. & MENARD, R. 1996. Recent insights into cysteine protease specificity: Lessons for drug design. *Perspect. Drug Des.*, 6, 33-46.
- THUNIG, J., HANSEN, S. H. & JANFELT, C. 2011. Analysis of Secondary Plant Metabolites by Indirect Desorption Electrospray Ionization Imaging Mass Spectrometry. *Anal Chem* 83, 3256-3259.
- TURK, V., STOKA, V., VASILJEVA, O., RENKO, M., SUN, T., TURK, B., *et al.* 2012. Cysteine cathepsins: From structure, function and regulation to new frontiers. *BBA – Proteins and Proteomics.* 1824, 68-88.
- VINCENT, J. L. & BREWIN, N. J. 2000. Immunolocalization of a Cysteine Protease in Vacuoles, Vesicles, and Symbiosomes of Pea Nodule Cells. *Plant Physiol.* 123, 521-530.
- WANG, W., TAI, F. & CHEN, S. 2008. Optimizing protein extraction from plant tissues for enhanced proteomics analysis. *J. Sep. Sci.*, 31, 2032-2039.
- WANG, Z. & BENNING, C. 2012. Chloroplast lipid synthesis and lipid trafficking through ER-plastid membrane contact sites. *Biochem Soc Trans.* 40, 457-463.
- WEIER, E. 1936. The structure of the non-starch-containing beet chloroplast. *Am J Bot.*, 23, 645-652.
- WIEDERANDERS, B., KAULMANN, G. & SCHILLING, K. 2003. Functions of propeptide parts in cysteine proteases. *Curr Protein Pept Sci.*, 4, 309-326.
- YANG, H., BARROS-RIOS, J., KOURTEVA, G., RAO, X., CHEN, F., SHEN, H., *et al.* 2017. A re-evaluation of the final step of vanillin biosynthesis in the orchid *Vanilla planifolia*. *Phytochemistry.* 139, 33-46.
- ZAPROMETOV, M. N. & NIKOLAEVA, T. N. 2003. Chloroplasts isolated from kidney bean leaves are capable of phenolic compound biosynthesis. *Russ J Plant Physiol.* 50, 623-626.
- ZENK, M. H. 1965. Biosynthese von vanillin in *Vanilla planifolia* Andr. . *Z Pflanzenphysiol* 53, 404-414.

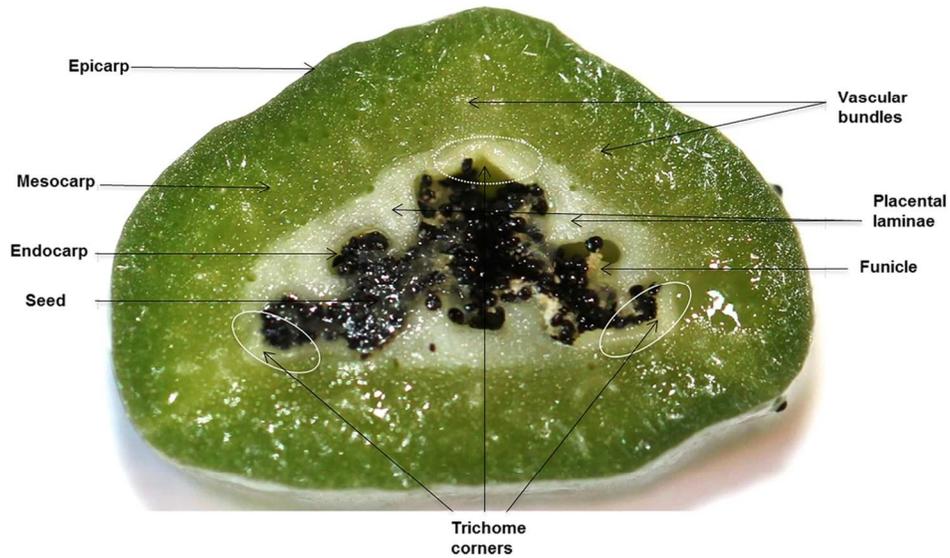


Figure 1: Transverse section of a six-months-old vanilla pod with arrows pointing to the different tissues present.

119x67mm (300 x 300 DPI)

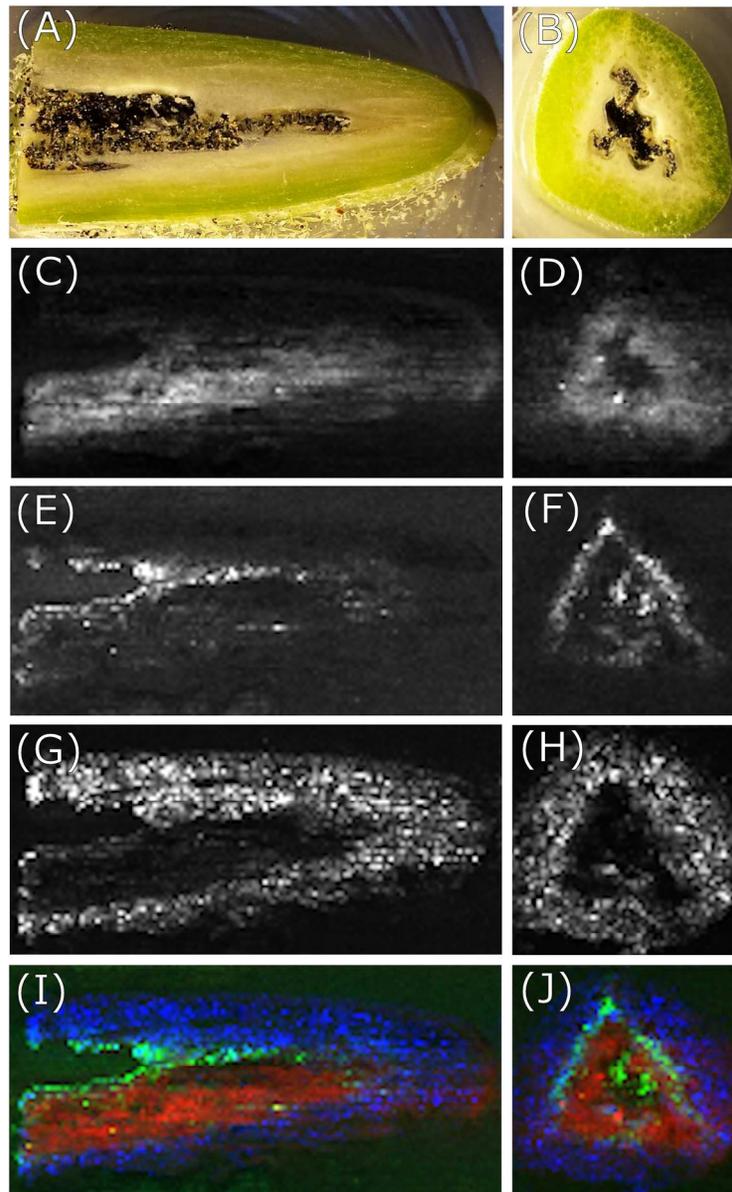


Figure 2: DESI-MSI obtained images of longitudinal (left column) and cross (right column) sections of a six-months-old vanilla pod.

Panels A and B: Photo of vanilla pod tissue obtained immediately following longitudinal and cross cryo-sectioning, respectively.

Panels C and D: Vanillin distribution (m/z 153)

Panels E and F: Vanillin glucoside distribution (m/z 337)

Panels G and H: Sucrose distribution (m/z 381)

Panels I and J: Colored overlay of vanillin (red), vanillin glucoside (green) and sucrose (blue) distributions.

192x311mm (300 x 300 DPI)

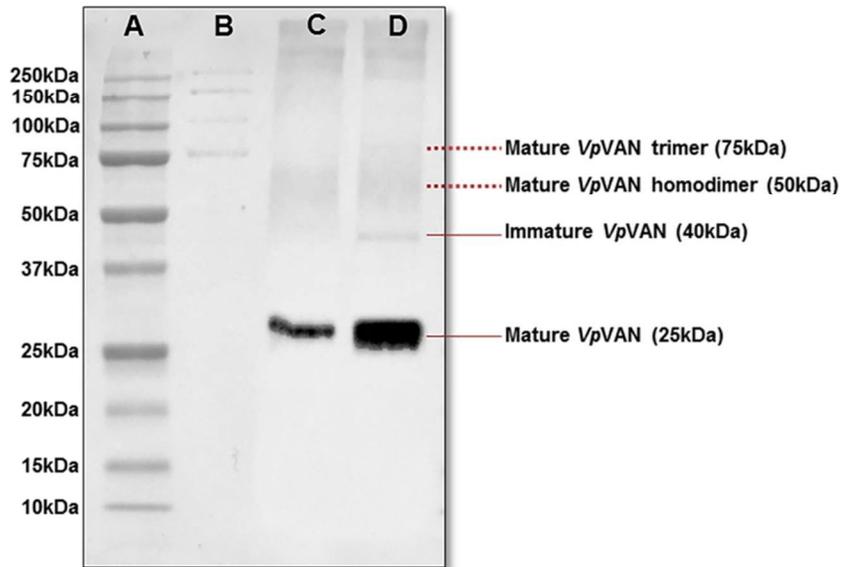


Figure 3: Western blot analysis of the presence of mature and immature forms of VpVAN in protein extracts of vanilla pods and in *N. benthamiana* leaves following transient expression of VpVAN.

Lane (A): Pre-stained protein ladder Bio-Rad

Lane (B): Un-stained protein ladder Bio-Rad

Lane (C): Protein extract from *N. benthamiana* leaves following transient expression of VpVAN probed with an antibody specific to the C-terminal sequence of VpVAN.

Lane (D): Protein extract of a seven-months-old vanilla pod probed with an antibody specific to the C-terminal sequence of VpVAN.

10 μ g of protein were applied to each lane.

82x48mm (300 x 300 DPI)

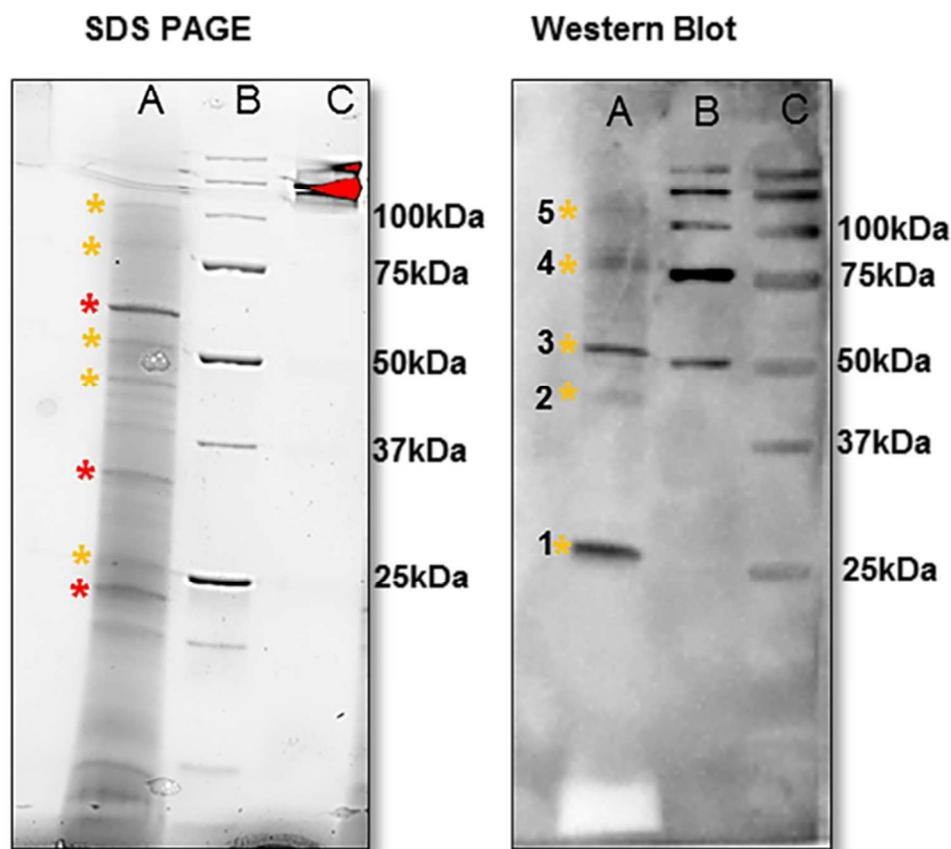


Figure 4: Mature *VpVAN* homodimer and putative oligomers detected in the crude *V. planifolia* extracts as demonstrated by SDS-PAGE followed by Western blot analysis. !! + Lane (A): Crude protein extract from a seven-months-old vanilla pod from *V. planifolia* !! + Lane (B): Pre-stained protein ladder Bio-Rad!! + Lane (C): Un-stained protein ladder Bio-Rad!! + 1: *VpVAN* mature protein (25kDa) 2: *VpVAN* immature protein (39kDa) 3: Putative mature *VpVAN* homodimer (50kDa) 4: Putative mature *VpVAN* trimer (75kDa) 5: Putative mature *VpVAN* oligomers (100kDa)!! + !! + The proteins present in a seven-months-old vanilla pod were separated by SDS-PAGE (12% Criterion™ TGX Stain-Free Precast Gels) and visualized using ChemiDoc MP Imaging System (Bio-Rad, US). The major protein components present in the pod extracts are marked with * (red) while *VpVAN* and putative oligomers are marked with * (yellow). Western blot analysis was conducted using a C-terminal specific antibody against *VpVAN* in 1:50 dilution. At this extremely high antibody concentration, the primary antibody demonstrated specificity towards *VpVAN*. No cross reactions were observed with the major protein components present in the extract.!! +

75x66mm (300 x 300 DPI)

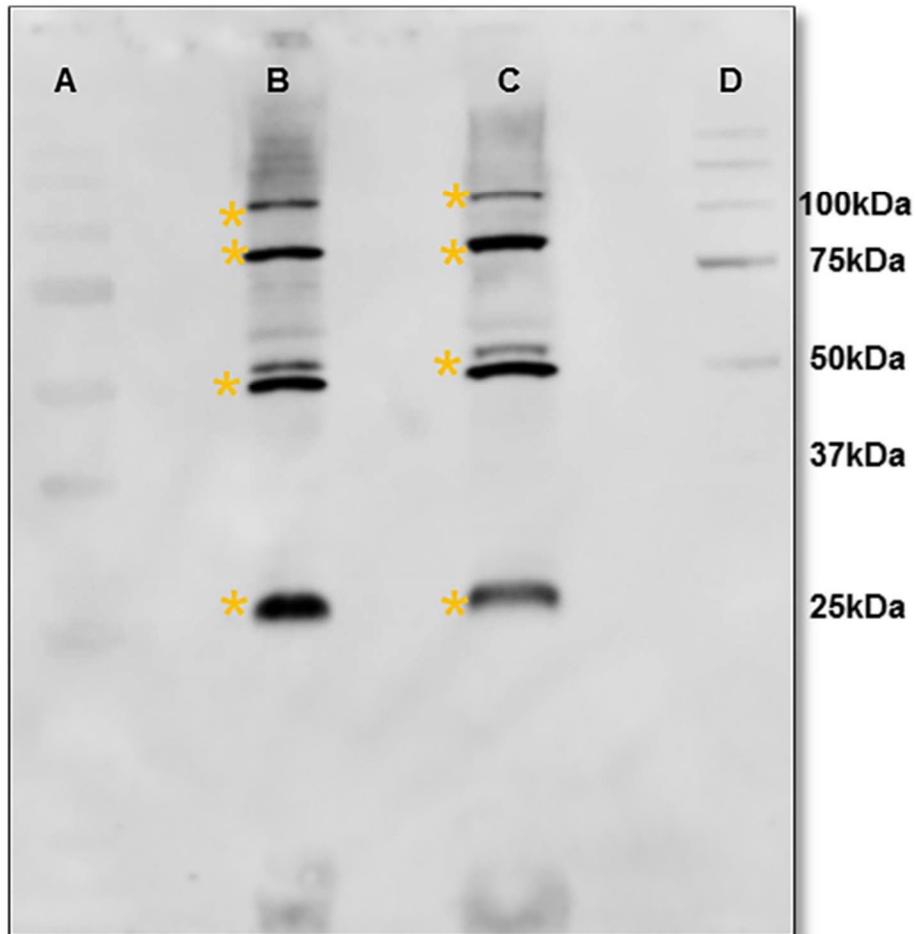


Figure 5: The ratio between monomeric and putative oligomeric forms of *VpVAN* as analysed by treatments with reductant and oxidant as monitored by SDS-PAGE followed by Western blot analysis. !! + Crude protein extracts from eight-months-old pods were analyzed. Protein bands recognized by the *VpVAN* antibody are marked with * (yellow).!! + Lane A: Pre-stained protein ladder Bio-Rad!! + Lane B: Treatment with 50mM TCEP for 15 min !! + Lane C: Treatment with 50 mM TCEP for 15 min followed by 50mM potassium fericyanide for 30 min !! + Lane D: Un-stained protein ladder Bio-Rad!! +

81x78mm (300 x 300 DPI)

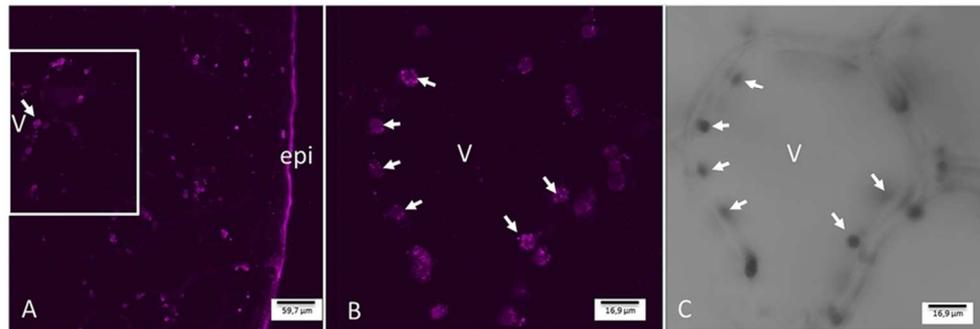


Figure 6: Immunohistochemical localization of *VpVAN* in cytoplasmic organelles in a seven-months-old *V. planifolia* pod as observed by confocal microscopy. !! + Panel A: Transverse section of a seven-months-old *V. planifolia* pod providing overview of *VpVAN* localization in the mesocarp. !! + Panel B: Close up of a single mesocarp cell showing the intra-cellular localization of *VpVAN* in plastids in the cytoplasm. !! + Panel C: The same section as shown in panel B observed with translucent light.!! + Images were obtained from transverse section of a seven-months-old *V. planifolia* pod using the *VpVAN* C-terminal antibody and a goat-anti rabbit antibody labeled with fluorescein isothiocyanate (FITC). The images were recorded using a Leica SPII confocal scanning microscope. Arrows indicate position of selected cytoplasmic plastids harboring *VpVAN*. !! + Abbreviations: epi: epicarp, v: vacuole!! +

81x27mm (300 x 300 DPI)

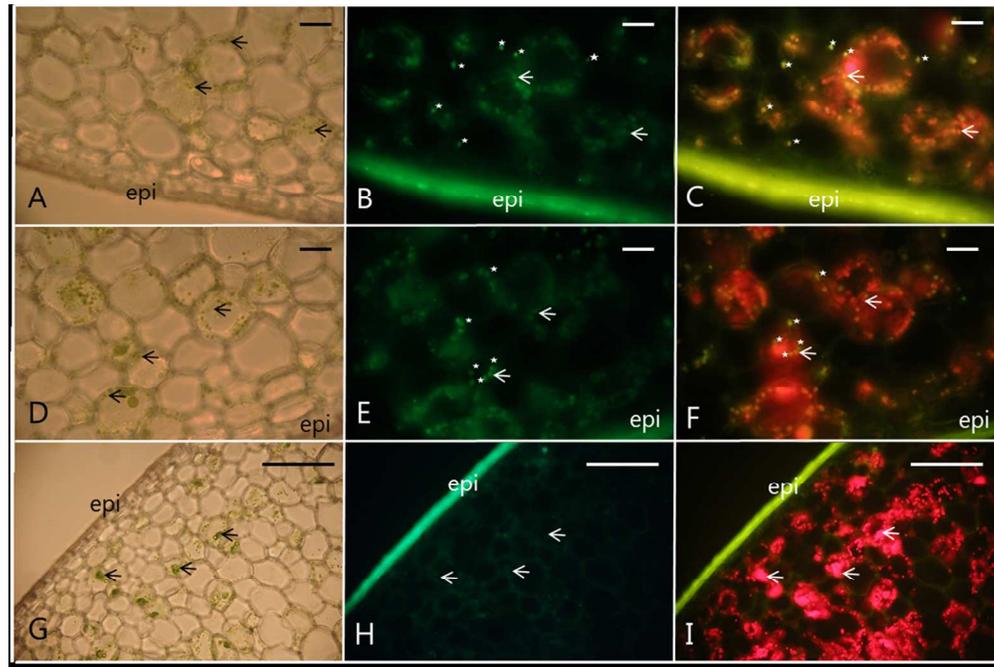


Figure 7: Immunolocalization of *VpVAN* to chloroplasts and phenyloplasts in seven-months-old *V. planifolia* pods using an antibody specific to the C-terminal sequence of *VpVAN*.

Panels A-F: Data obtained from two transverse sections of two different pods are shown; *V. planifolia* pod 1:

Panels A to C and *V. planifolia* pod 2: Panels D to F

Panels A, D and G: Visualization of the examined transverse vanilla pod sections using light microscopy documenting the localization of chloroplasts. Chloroplasts are identified based on their chlorophyll content. Selected chloroplasts are marked with black arrows.

Panels B and E: Immuno-detection of *VpVAN* in chloroplasts and phenyloplasts by FITC and fluorescence microscopy. The chloroplasts and phenyloplasts are stained green due to the antibody reaction. Selected phenyloplasts and chloroplasts are marked with white stars and white arrows, respectively.

Panels C and F: Localization of *VpVAN* in chloroplasts (white arrows) and phenyloplasts (white stars) as monitored by fluorescence microscopy using a filter setting enabling simultaneous detection of FITC and chlorophyll fluorescence. Chlorophyll fluorescence appears in red and demonstrates the localization of chloroplasts. Phenyloplasts that are totally free of chlorophyll appear in green on panel C and F while those plastids retaining a small amount of chlorophyll due to incomplete re-differentiation appear in yellow on panels C and F.

Panels G, H and I: Control panels, vanilla pod sections probed with pre-immune serum. No cross-reactions were observed.

Chloroplasts: Black arrows (A, D and G) and white arrows (B, C, E, F, H and I); Phenyloplasts: white stars. The same selected set of chloroplasts and phenyloplasts is labelled on the different panels to facilitate interpretation of the nature of the observed structures.

Scale bars correspond to 100 μ m. Abbreviations: epi: epicarp.

119x80mm (300 x 300 DPI)

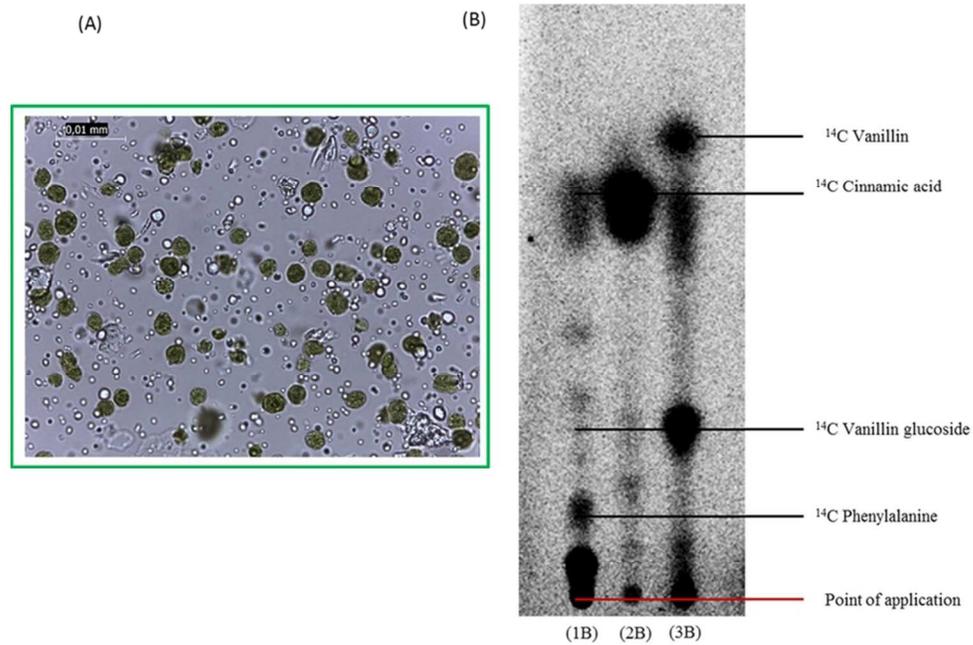


Figure 8: Intact chloroplasts isolated from eight-months-old *V. planifolia* pods de novo synthesize vanillin.!! † Panel A: The purity and integrity of the isolated chloroplasts as monitored by light microscopy. !! † Panel B: Vanillin biosynthetic activity in isolated chloroplasts as monitored by administration of [^{14}C]-phenylalanine (lane 1B) and [^{14}C]-cinnamic acid (lane 2B) as substrates. An extract from the inner part of a *V. planifolia* pod disc incubated with [^{14}C]-vanillin was applied (lane 3B) to visualize the migration position of [^{14}C]-vanillin glucoside.

78x51mm (300 x 300 DPI)