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Production of enzymes for the removal of odorous substances in plant biomass

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ABSTRACT

Residual plant biomass collected from agricultural, technical or biopharmaceutical processes contains odorous substances. The latter are often unacceptable for customers if the biomass is used in sustainable products such as building materials, paints, glues or flame-resistant foils. The objective of this study was to identify enzymes that can prevent the formation or facilitate the degradation of odorous substances such as butanol, eugenol or ethyl acetate and their derivatives in residual biomass. We used plant cell packs (PCPs) as a small-scale screening platform to assess the expression of enzymes that break down odorous substances in tobacco biomass. First, we compiled a list of volatile compounds in residual plant biomass that may give rise to undesirable odors, refining the list to 10 diverse compounds representing a range of odors. We then selected five monomeric enzymes (a eugenol oxidase, laccase, oxidase, alkane mono-oxidase and ethyl acetate hydrolase) with the potential to degrade these substances. We transiently expressed the proteins in PCPs, targeting different subcellular compartments to identify optimal production conditions. The maximum yield we achieved was ~20 mg kg^{-1} for *Trametes hirsute* laccase targeted to the chloroplast. Our results confirm that enzymes for the removal of odorous substances can be produced in plant systems, facilitating the upcycling of residual biomass as an ingredient for sustainable products.

Authors' contributions/authors statement

JB, PO and MK designed the experiments. PO and MK performed the experiments and analyzed the data. JB supported the statistical analysis. PO and JB and wrote the manuscript. JB revised the manuscript and secured funding for the project.

1. Introduction

The environmental impact of manufacturing processes is an increasing concern, especially when such processes are heavily reliant on petrochemical resources. Efforts to transition from linear to circular manufacturing have encouraged the development of biotechnological production processes using cell factories based on engineered microbes and renewable resources such as spent media or straw hydrolysate [1–3], but the underlying processes can still have a large carbon footprint. For example, when recombinant enzymes are produced by microbial fermentation, 1–2 kg of carbon dioxide (CO_2) is released per kilogram of cell dry mass, and up to 20 kg of CO_2 is released per kilogram of enzyme product [4].

The carbon footprint of biotechnology can be reduced by recycling waste streams such as extracted biomass and spent media [1]. Plants can be regarded as self-building, single-use biodegradable reactors that fix CO_2 during growth, resulting in a lower footprint than most cell-based host systems. However, the bulk of the plant biomass is discarded in most current plant molecular farming applications, ultimately releasing this fixed CO_2. The environmental impact of such processes will increase as they are scaled up and rolled out to include commodity products

Abbreviations: CaMV, cauliflower mosaic virus; CHS, chalcone synthase; ER, endoplasmic reticulum; OD_{600nm}, optical density at 600 nm; PCP, plant cell pack; UTR, untranslated region.

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rather than niche biopharmaceuticals [5,6].

Several options to make use of residual biomass have been proposed, including the development of biobased cosmetic ingredients and building materials [7]. However, one significant drawback that hinders such cascading biomass applications is the accumulation of odorous substances such as 1-butanol or geosmin [8,9], which evaporate from residual plant material over time and from derived recycled products. Odorous biomass is incompatible with the requirements of recycling companies and the expectations of end-users [10,11] and may even attract disease vectors [12,13]. One way to address this challenge is the co-expression of enzymes that prevent the formation or catalyze the degradation of odorous volatiles, thus facilitating the upcycling of residual plant biomass once a primary product has been extracted. The co-expression of enzymes with a primary technical or pharmaceutical protein has already been demonstrated, including enzymes that introduce desirable post-translational modifications [14,15], enzymes that remove proteases or other plant cell components that reduce the quality, quantity or accessibility of a primary product [16], and enzymes that degrade cell-wall polysaccharides to facilitate biomass processing [17].

Here, we screened the literature to identify odorous substances that are typically found in spent plant biomass and enzymes that can break down these compounds and/or their precursors. We then cloned sequences representing five such enzymes and introduced them into plant expression vectors. Finally, we expressed the enzymes in plant cell packs (PCPs) and quantified their accumulation in different cellular compartments. The latter is important because pH and redox potentials differ between such compartments. For example, as opposed to the cytosol, endoplasmic reticulum (ER) and apoplast provide oxidizing conditions that allow the formation of disulfide bonds that are important for the structure of several groups of proteins [18,19]. Similarly, protein glycosylation is carried out in the ER (basal glycosylation) and Golgi apparatus (trimming and modifications) [20]. Accordingly, substantial differences in enzyme stability have been reported for different plant cell compartments [21,22]. Similar results have been found for various other proteins too, e.g. antibodies and fluorescent proteins too [23–27] xxx. The targeting to the individual compartments is controlled by leader sequence or tags, and there are many variants for each of them [28–33]. The resulting sub-cellular targeting can be predicted based on the protein amino acid sequence using bioinformatic tools like LOCALIZER or TargetP [34,35].

We found that all five enzymes were expressed in at least one compartment and often in more, confirming that the co-expression of odor-removing enzymes is a viable strategy to improve the downstream utilization of spent plant biomass. In this explorative study, we used PCPs as a rapid screening tool to identify promising conditions for enzyme synthesis in plants. In an applied setting in the future, expression can be carried out in different transgenic plants using, for example, inducible promoters to avoid interference with plant growth, development and (primary) product formation [36] but also to reduce the handling efforts (e.g. infiltration of every plant in a transient setting).

2. Materials and methods

2.1. Expression vectors

The nucleotide sequences of five enzymes were codon-optimized for *Nicotiana* spp. and synthesized (Thermo Fisher Scientific, Waltham, MA, USA) with flanking N-terminal *Bsp*HI or *Pci*I sites and C-terminal *Not*I sites and a C-terminal His_6 tag. The sequences were trimmed with the same enzymes (New England Biolabs, Ipswich, MA, USA), separated by 1.2 % (m v^{-1}) agarose gel electrophoresis and purified using a NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) before insertion into the plant expression vector pTRAc. Four variants were created for each gene to target the corresponding enzyme to the apoplast, chloroplast, cytosol or endoplasmic reticulum (ER) (Table S1; supplementary file "odor_enzymes_opdensteinen_et_al_gene_seq_v1"). The native genes were of bacterial origin and did not contain any targeting signals or leader peptides. The double enhanced cauliflower mosaic virus (CaMV) 35S promoter [37] was combined with the *Petroselinum crispum* chalcone synthase (CHS) 5′ untranslated region (UTR) [38] and the CaMV 35S polyadenylation site/terminator [39] in all constructs to maximize gene expression. The assembled vectors were propagated in *Escherichia coli* DH5α cells (New England Biolabs) grown in lysogeny broth (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride, 50 mg L^{-1} ampicillin, pH 7.0) at 37 °C for 24 h, shaking at 160 rpm. *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*) strain GV3101pMP90RK was transformed with each vector by electroporation as previously described [40].

2.2. Cultivation of R. radiobacter

The *R. radiobacter* cultures were grown in Riplate 96 deep-well round-bottom plates (Ritter, Schwabmünchen, Germany) as previously described [22]. Briefly, 500 µL plant peptone *Agrobacterium* medium (PAM4) [41] containing 25 mg L^{-1} kanamycin, 25 mg L^{-1} rifampicin and 50 mg L^{-1} carbenicillin was inoculated to an optical density at 600 nm (OD_{600nm}) of 0.04 with ~20 µL from the appropriate glycerol stock (25 % v v^{-1} glycerol; OD_{600nm} of 1.0) and cultivated at 28 °C shaking at 1000 rpm (4 mm eccentricity). To achieve a homogenous OD_{600nm} at harvesting, a 500-µL subculture was inoculated (OD_{600nm} of 0.1) from the starting culture after 24 h using the same medium and cultivation conditions [22]. Plates were covered with a gas-permeable membrane (water vapor transmission rate = 4200 g m^{-2} d^{-1}) during incubation [22]. After a further 24 h, the cultures were centrifuged (3800×*g*, 5 min, ~22 °C) and the pellets were resuspended in infiltration buffer (0.5 g L^{-1} Murashige and Skoog salts, 50 g L^{-1} sucrose, 2 g L^{-1} glucose monohydrate, 15 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 200 µM acetosyringone, pH 5.6) and adjusted to a final OD_{600nm} of 0.4 with infiltration buffer. The resulting suspension was incubated on a 50-rpm rotary shaker at 22 °C for 1 h to induce *vir* gene expression [42].

2.3. Protein expression in PCPs

PCPs were cast from *Nicotiana tabacum* BY-2 cell suspension cultures grown in MS medium (4.3 g L^{-1} Murashige and Skoog major and minor salts, 0.1 g L^{-1} myo-inositol, 0.001 g L^{-1} thiamine, 0.0002 g L^{-1} 2,4-dichlorophenoxyacetic acid and 0.2 g L^{-1} potassium phosphate, pH 5.8) that contained 30 g L^{-1} sucrose or glucose as carbon source. The cells were grown in 200-mL Erlenmeyer glass flasks for 7 days at 160 rpm (eccentricity of 50 mm), 50% relative humidity and 26 °C until reaching a wet biomass of ~200 g L^{-1} [43]. PCPs were cast using an automated protocol by transferring 300 µL cell suspension to AcroPrep Advance PP/PE multi-well filter plates with a pore size of 30–40 µm (Pall, Dreieich, Germany) followed by centrifugation (1800×*g*, 1 min, 20 °C) to remove the culture medium [22]. PCPs were infiltrated by transferring 100 µL of the *R. radiobacter* suspension (OD_{600nm} = 0.4) onto individual PCPs, followed by incubation for 1 h at 22 °C before removing the liquid by centrifugation (1600×*g*, 1 min, 22 °C). The infiltrated PCPs were then incubated for 72 h at 26 °C and a relative humidity of 80 % as previously described [22].

2.4. Protein extraction from plant cell packs

Proteins were extracted from PCPs using a 3 v m^{-1} ratio of extraction buffer (50 mM disodium phosphate dihydrate, 500 mM sodium chloride, 10 mM sodium metabisulfite, pH 8.0) in a MM 300 beadmill (Retsch, Han, Germany) as previously described [22]. Briefly, PCPs and one chrome bead per PCP were transferred into Chromabond collection tubes (Macherey-Nagel), sealed with PTFE-coated silicone mats and extracted for 2 × 3 min at 28 Hz. Plates were inverted following the first extraction cycle. Extracts were clarified by centrifugation (5100×*g*, 8 min, 4 °C) and supernatants were stored at −20 °C.

2.5. LDS-PAGE and gel staining

NuPAGE 4–12 % Bis-Tris LDS-PAGE gels (Thermo Fisher Scientific) with 26 gel slots were used according to the manufacturer's instructions. Samples containing 1 × reducing agent (Thermo Fisher Scientific) and 1 × LDS loading buffer (Thermo Fisher Scientific) were denatured for 10 min at 70 °C on a Techne shaking Dri-Block DB-3 heat block (Bibby Scientific, Stone, UK) or alternatively on a Biometra-TRIO PCR cycler (Biometra, Göttingen, Germany). We loaded 10 μL of denatured sample or 5 μL PageRuler pre-stained protein standards 10–180 kDa (Thermo Fisher Scientific) per gel slot. Electrophoresis was carried out in a SureLock Tandem Midi-Gel-Tank (Thermo Fisher Scientific), using a PowerPac HC power supply unit (Bio-Rad Laboratories, Hercules, CA, USA) at 200 V for 37 min. Gels were washed for 15 min with deionized water and stained with SimplyBlue SafeStain (Thermo Fisher Scientific) for 1 h at 22 °C on a rotary shaker (Bibby Scientific) at ~20 rpm. After 1–2 h of destining in deionized water, gels were transferred onto a plastic foil and scanned using a CanoScan 5600F (Canon, Tokyo, Japan) film scanner and Adobe Photoshop CS5.1 (Adobe, San Jose, USA) at 600 dots per inch.

2.6. Western blotting

Western blotting, dot blots and immunostaining were carried out as previously described [24,25]. For dot blots, 5 μL of PCP extract clarified by centrifugation (5100×*g*, 8 min, 4 °C) was applied to Amersham Protran 0.2 μm nitrocellulose membranes (GE Healthcare) along with 5 μL of His_6-tagged DsRed at concentrations of 0.5, 2.0, 4.0, 6.0, 8.0, 10.0, 12.5 and 15.0 mg L^{-1}. Target protein concentrations were estimated based on densitometric analysis using specific antibodies (Table 1).

2.7. Bradford assay

The concentration of total soluble protein (TSP) in PCP extracts was quantified using the Bradford method with triplicate 5-μL samples in transparent 96-well flat-bottom microplates (Greiner Bio One, Kremsmuenster, Austria) [44]. Triplicates of 5 μL bovine serum albumin (BSA) at concentrations of 2000, 1500, 1000, 750, 500, 250, 125 and 0 mg L^{-1} were used as quantitation standards. We mixed 195 μL of Coomassie Protein Assay reagent (Thermo Fisher Scientific) with each sample, incubated the plates for 10 min at 22 °C in the dark and measured the absorbance at 595 nm twice for each well using a Synergy H1 plate reader (BioTek, Winooski, USA). Data were exported using the Gen5 software v3.10.06 (BioTek).

2.8. Mass spectrometric analysis of odorous compounds in Nicotiana benthamiana residual biomass

Seeds were a donation from the RWTH Aachen University in 2006. *N. benthamiana* seeds were germinated on stone wool blocks soaked with 1.0 g L^{-1} Ferty 2 Mega fertilizer solution (Planta Düngemittel, Regenstauf, Germany) and incubated for 7 days before transfer to custom-made plastic trails and incubation in a greenhouse setting at 25/22 °C light/dark and ~70 % relative humidity with a ~14 h photoperiod. Natural light was augmented using 400-W IP65 sodium discharge lamps (Phillips, Amsterdam, Netherlands) and SON–K artificial light (Phillips) if required, depending on the weather conditions [45,46]. The plants were irrigated with 1.0 g L^{-1} Ferty 2 Mega fertilizer solution adjusted to pH 5.8 for 12 min ~2–4 times per day using an ebb-and-flow hydroponic system that removed residual liquid after each watering phase. Plants were harvested 42 days after seeding, homogenized in a blade-based blender and residual solids recovered from a bag filter as described before [24,47] and then frozen at −80 °C until analysis. About 1 g of thawed solids was transferred into a 20-mL headspace vial, incubated for 15 min at 30 °C and 1 mL of gas phase was subjected to gas chromatographic analysis on a 7890B GC with 7000C MS (Agilent, Santa Clara, USA) and MPS2 autosampler (GERSTEL GmbH & Co. KG, Mülheim an der Ruhr, Germany) coupled with mass spectrometry using a 30-m Rtx-1701 (medium polarity) column with a 0.5 μm film thickness and a 0.25 μm inner diameter. Resulting candidate spectra were matched against the NIST library (https://webbook.nist.gov/chemistry/name-ser/) [48]. Additional aliquots of thawed solids were incubated for 72 or 168 h at 25 °C and then subjected to the same analysis.

2.9. Statistical analysis

Expression data representing PCPs prepared from BY-2 cells cultivated in medium containing glucose or sucrose were compared using a paired *t*-test with each expression vector (i.e., combination of enzyme and subcellular localization) as an element of the sample. Because differences between two samples were compared, the equality of variances was not relevant for the comparison [49]. However, we also applied the paired *t*-test to $\log_{10}$ transformed data to compensate for non-normal distribution.

3. Results and discussion

3.1. Odorous substances in plant biomass and its derivatives

More than 1700 individual compounds are listed in the Leibniz-LSB@TUM Odorant Database as of May 2023 (https://www.leibniz-lsb.de/datenbanken/leibniz-lsbtum-odorant-database/odorantdb) so it was beyond the scope of this study to compile an exhaustive list of molecules present in residual plant biomass or processing waste. Instead, we screened the literature in databases such as PubMed (https://pubmed.ncbi.nlm.nih.gov/) using query strings such as "plant biomass odorous substance" or "plant biomass odorous compound". We found ~70 publications, 50 % of which were open access or otherwise available to us, and from those articles we selected documents reporting odorous substances present in plant biomass (e.g., algae, garlic, tea, tobacco, tree leaves or wood), processing streams or waste water (Table 2). In addition, we identified 5 substances present in the solid residuals after protein extraction from *N. benthamaina* (Table 2 and S2). Whereas *Nicotiana* spp. are known to contain various secondary metabolites [50,51], none of the five compounds we identified was found in the available secondary metabolite networks of *N. tabacum* or *N. sylvestris* available at the Kyoto Encyclopedia of Genes and Genomes (KEGG; a network for *N. benthamiana* is currently not available) [52]. It appeared unlikely to us that plant cultivation conditions, e.g. high nitrate concentrations [53], were responsible for a sudden onset of a synthesis of the compounds. Instead, we think that they were a product of enzymatic degradation of unsaturated fatty acids, e.g. by lipoxygenase released during plant tissue homogenization, and subsequent oxidation. A similar mechanism has been described for the development of (volatile) off-flavors in pulses (e.g. lupins) that are caused by hexanals and furans [54], the same type of compounds we found in our analysis.

We clustered the substances according to their chemical type and manually selected 10 of them to form a target compound set covering

Table 1
Primary and secondary antibodies used for immunostaining.

Antibody	Abbreviation	Conjugate	Manufacturer	Used dilution	Concentration [mg L^{-1}]
Rabbit anti-His_6	MonoRab	None	GenScript (A00174)	1:5000	0.10
Goat anti-rabbit IgG	GAR	Alkaline phosphatase	Jackson (111-045-045)	1:5000	0.06

Table 2
Odorous compounds reported in plant biomass and its derivatives.

#	Compound name[a,b]	Chemical formula	Type	Mass [Da]	Boiling point [°C]	Source	Odor[c]	Ref.
1	***1-Butanol***	**C4H10O**	**Alcohol**	**74**	**118**	**Wood**	**Malty, solvent-like**	[8]
2	*2-Propanol*	(CH3)2CHOH	Alcohol	60	83	Solid wastes	Pungent	[55]
3	*cis*-3-hexen-1-ol	C6H12O	Alcohol	100	157	Drinking water	Green, grassy	[56]
4	Hexanol	C6H14O	Alcohol	102	157	Processed foods	Grassy, marzipan-like	[57]
5	Furfural	C5H4O2	Aldehyde	96	162	Processed foods	Sweet, cereal-like	[57]
6	n-Hexanal	C6H12O	Aldehyde	100	130	Processed foods	Green, grassy	[57]
7	*(E)-2-Hexenal*	C6H10O	Aldehyde	98	146	*N. benthamiana*	Green-apple-like	This study
8	***n-Pentanal***	**C5H10O**	**Aldehyde**	**86**	**102**	**Lakes; processed foods**	**Green, fatty, moldy**	[57, 58]
9	*Cyclohexane*	C6H12	Alkane	84	81	Solid wastes	Gas-like, rubber-like for derivatives	[55]
10	Dodecane	C12H26	Alkane	170	214	Solid wastes	[Gasoline-like to odorless]	[55]
11	Pentane	C5H12	Alkane	72	36	Solid wastes	[Gasoline-like]	[55]
12	1-Octene	C8H16	Alkene	112	121	Solid wastes	[Mushroom-like]	[55]
13	Diphenylether	(C6H5)2O	Aromatic ether	170	259	Drinking water	[Geranium-like]	[56]
14	1,3-Dimethyl-Benzene	C8H10	Aromatic	106	139	Wood	[Metal-like]	[8]
15	*2-Ethylfuran*	C6H8O	Aromatic	96	92	*N. benthamiana*	Ethereal, rum, cacao	This study
16	3,4-Dihydro-8-hydroxy-3-methyl-1H-2-benzopyran-1-one	C10H10O3	Aromatic	178	n.a.	Wood	[Cork-like]	[59]
17	3-Pyridinol	C5H5NO	Aromatic	95	129	Tea	[Fruity]	[60]
18	Cinnamic acid	C9H8O2	Aromatic	148	300	Tobacco	[Fruity]	[61]
19	*Ethylbenzene*	C8H10	Aromatic	106	136	Solid wastes	Terpene-like	[55]
20	***Eugenol***	**C10H12O2**	**Aromatic**	**164**	**253**	**Diverse plants**	**Smoky, clove-like**	[62]
21	*Quinoline*	C9H7N	Aromatic	129	237	Tobacco	Mint-like, rubber-like	[61]
22	Styrene	C8H8	Aromatic	104	145	Drinking water	[Sweet]	[56]
23	***Cyclohexanol***	**C6H12O**	**Cyclic alcohol**	**100**	**161**	***N. benthamiana***	**Ethereal, fruity**	This study
24	***Geosmin***	**C12H22O**	**Cyclic alcohol**	**182**	**270**	**Algae**	**Musty, earthy, beetroot-like**	[9]
25	2-Formylpyrrole	C5H5NO	Cyclic ketone	95	217	Tea	[Musty]	[60]
26	*cis*-3-Hexenyl acetate	CH3CO2CH2CH2CH═CHC2H5	Ester	142	75	Drinking water	Green banana-like	[56]
27	***Ethyl acetate***	**C4H8O2**	**Ester**	**88**	**77**	**Wood**	**Irritating, glue-like**	[8]
28	*1-Hepten-3-on*	C7H12O	Ketone	112	42	*N. benthamiana*	Geranium-like	This study
29	2-butanone	C4H8O	Ketone	72	80	Solid wastes	Ethereal, fruity	[55]
30	*3-Pentanon*	C5H10O	Ketone	86	102	*N. benthamiana*	Gasoline, fruity	This study
31	Acetone	C3H6O	Ketone	58	56	Solid wastes	Solvent-like, pungent	[55]
32	Allicin	C6H10OS2	Organosulfur	162	[decomposes]	Garlic	[Sweet]	[63]
33	Diallyl disulfide	C6H10S2	Organosulfur	146	180	Garlic	Garlic-like	[63]
34	Dimethyl disulfide	C2H6S2	Organosulfur	94	110	Drinking water	Cabbage-like, sulfuric	[56]
35	*2-Methylisoborneol*	C11H20O	Terpene or terpenoid	168	n.a.	Algae	Moldy, musty	[9]
36	Limonene	C10H16	Terpene or terpenoid	136	176	Tree leaves	Citrus-like	[55]

[a] Names in bold indicate compounds potentially broken down by the enzymes selected for testing in this study.
[b] Italicized compounds are abundant in relevant sources.
[c] The odor type was retrieved from the Leibniz-LSB@TUM Odorant Database (https://www.leibniz-lsb.de/datenbanken/leibniz-lsbtum-odorant-database/odor-antdb/; accessed between 2022-07–15 and 2023-05-15). If other sources such as the cited references were used, the information is enclosed in brackets. Additional information about the compounds identified in *N. benthamiana* solids in this study can be found in Table S2.

Table 3
Enzymes found to convert odorous substances in plant biomass and its processing waste.

Compound name[a]	Type	Converting enzyme	UniProt ID	Mass [kDa]	Enzyme source organism	Native cellular localization	Substrate selectivity	Reaction product	Ref.
1-Butanol	**Alcohols**	**Alcohol oxidase**	**n.a.**	***16.5***	***Ochrobactrum* sp. AIU 033**	**n.a.**	**Diverse**	**Diverse**	[69]
2-Propanol	Alcohols	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n-Pentanal	**Aldehyde**	**Alkane 1-monooxygenase**	Q9WWW6	***46.1***	***Pseudomonas putida* (*Arthrobacter siderocapsulatus*)**	**Membrane associated**	**Diverse**	**Diverse**	[70]
Cyclohexane	Alkanes	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Ethylbenzene	Aromatics	Ethylbenzene dehydrogenase	Q5P5I0; Q5P5I1; Q5NZV0; Q5P5I3	*110.3; 39.7; 23.4; 25.4*	*Aromatoleum aromaticum* (strain EbN1) (*Azoarcus* sp. (strain EbN1))	Periplasmic space	Ethylbenzene	(S)-1-phenylethanol	[65]
Eugenol	**Aromatics**	**Eugenol oxidase**	Q0SBK1	***58.7***	***Rhodococcus jostii* (strain RHA1)**	**n.a.**	**Eugenol**	**Coniferyl alcohol**	[71]
Quinoline	Aromatics	Quinoline 2-oxidoreductase	P72222; P72223; P72224	*30.7; 18.0; 84.2*	*Pseudomonas putida*	n.a.	Quinoline	Quinolin-1(2H)-one	[66]
Geosmin	**Cyclic alcohol**	**Laccase**	Q02497	***55.7***	***Trametes hirsuta* (*Coriolus hirsutus*)**	**Secreted**	**Diverse**	**Diverse**	[72]
Ethyl acetate	**Ester**	**Ethyl acetate hydrolase**	Q8KQK1	***34.3***	***Pseudomonas putida***	**Cytoplasm**	**Diverse**	**Diverse**	**n. a.**[2]
2-Methylisoborneol	Terpene or terpenoid	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

[a] Entries in bold were used for subsequent expression analysis.[2] Enzyme identified in the BRENDA enzyme database (https://www.brenda-enzymes.org/; accessed between 2022-07–15 and 2023-05-15) [73].

both chemical diversity and a spectrum of odors (compounds highlighted in gray in Table 2).

3.2. Identification of enzymes that degrade plant odorous substances

Next, we conducted a second literature search to identify enzymes that use at least one of the 10 target compounds as a substrate (Table 3). In the case of 2-propanol, cyclohexene and 2-methylisoborneol, we did not retrieve specific enzymes but found reports of species or communities that can metabolize these compounds [64]. For ethylbenzene and quinoline, we identified a tetrameric dehydrogenase (UniProt IDs: Q5P5I0, Q5P5I1, Q5NZV0 and Q5P5I3; *Aromatoleum aromaticum* strain EbN1) [65] and a trimeric oxidoreductase (UniProt IDs: P72222, P72223 and P72224; *Pseudomonas putida*) [66], respectively. Although the balanced expression of polypeptide chains representing multimeric proteins is possible in plants [67], we decided that the lengthy selection process required to identify combinations of promoters, untranslated regions and other elements to achieve this outcome [68] was unsuitable for a proof-of-principle study. We therefore focused on monomeric enzymes, which we identified for the degradation of 1-butanol, *n*-pentanal, eugenol, geosmin and ethyl acetate.

3.3. Enzyme expression screening in PCPs

After selecting five candidate enzymes (bold in Table 3), we codon-optimized the coding sequences for *Nicotiana* spp. and inserted them into pTRA expression vectors [74] targeting the apoplast, chloroplast, cytosol or ER. The 20 expression constructs were then introduced into *R. radiobacter* (Table S1) for the infiltration of PCPs and transient expression [22,75]. We used either glucose or sucrose as the carbon source when cultivating BY-2 cells for the casting of PCPs because the carbon source and its concentration affect medium osmolality, which has a significant impact on subsequent transient protein expression in PCPs [43]. We found that protein expression was significantly higher in PCPs prepared from glucose-fed BY-2 cells compared to those grown on sucrose (paired *t*-test, untransformed data $p = 0.006$, $\log_{10}$ transformed data $p = 0.046$) (Fig. 1A), which was consistent with previous observations for proteins such as antibodies and ferritin [24,76]. Four of the proteins formed single bands at the anticipated masses indicating correct processing and no degradation by plant proteases (Fig. 1B). The latter is particularly relevant for future applications, such as enzyme activity in crude plant extracts and residual biomass. Exceptionally, the *Pseudomonas putida* ethyl acetate hydrolase formed four distinct bands when targeted to the chloroplast. The most intense band represented the expected mass of the protein, but we observed two smaller bands that could represent degradation products as well as a single larger band potentially representing a glycosylated variant or an unprocessed protein still carrying the transit peptide. The latter appeared more likely because the observed mass difference of an additional ~5–8 kDa matched well with the expected contribution of ~5.6 kDa by the rbcs leader peptide. The mass would also agree with that of 12 O-glycosylation (~0.5 kDa per site) and single N-glycosylation (~1.9 kDa per site [77]) that we identified in the protein sequence using "Glycosylation Predictor" [78]. However, the expected route of the protein containing the rbcs transit peptide is that of a direct import into the chloroplast without any detour to the ER (necessary for N-glycosylation) or Golgi (were the mass-relevant part of the O-glycosylation occurs) that would be required for the observed mass increase [79].

Interestingly, the *Ochrobactrum* sp. alcohol oxidase (for 1-butanol conversion) and the *Rhodococcus jostii* eugenol oxidase only accumulated at detectable levels when secreted to the apoplast (Fig. 1B). There was no information available in the literature to assess whether the extracellular space is the native compartment of these enzymes. For the other enzymes, we observed no correlation between the native subcellular localization and the accumulation in different plant cell compartments.

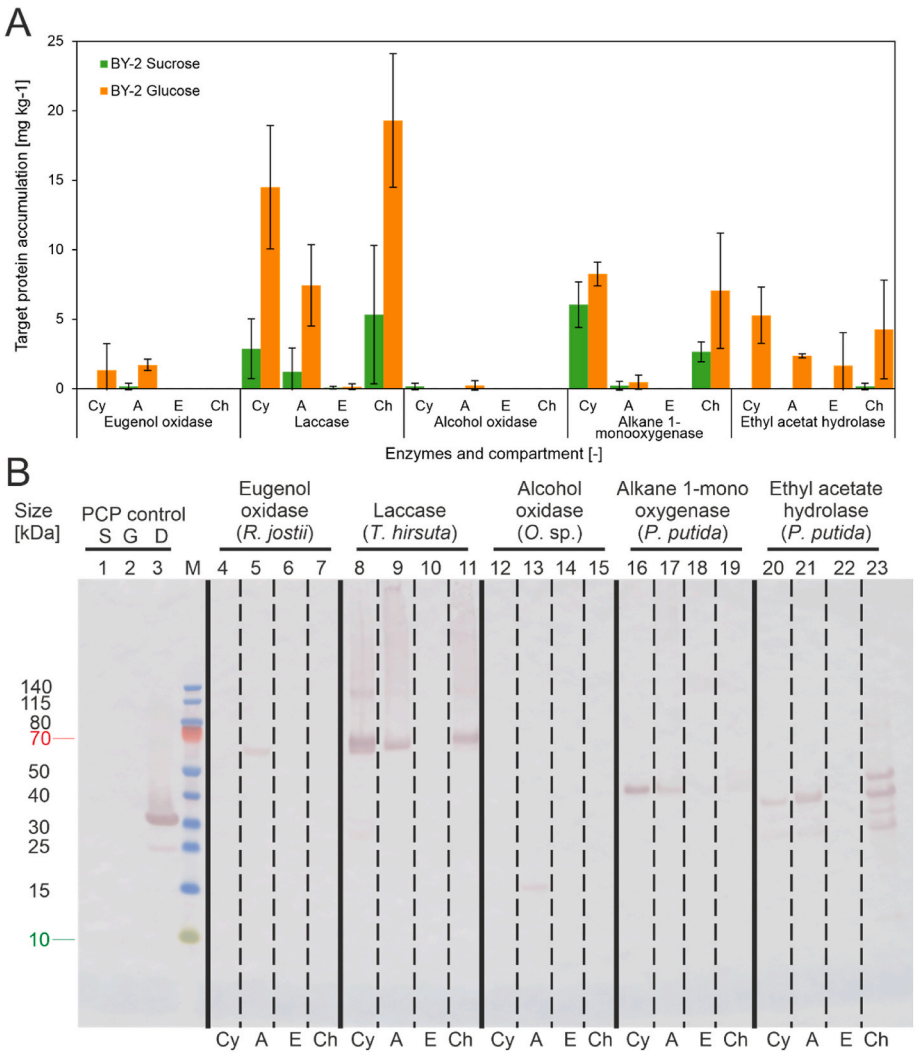

Fig. 1. Enzyme expression in plant cell packs. A. Quantitation of enzyme expression based on densitometric analysis of dot-blots (Figure S1) using defined DsRed standards. Data are means ± SD (n = 3 measurements in individual PCPs). B. PCP extracts separated by LDS-PAGE followed by blotting onto a nitrocellulose membrane probed using a combination of rabbit anti-His_6 antibodies and AP-conjugated goat anti-rabbit antibodies (Table 2). Samples were obtained from PCPs cast of BY-2 cells cultures on glucose containing medium. AP – alkaline phosphatase, LDS-PAGE – lithium dodecyl sulfate polyacrylamide gel electrophoresis, PCP – plant cell pack; compartments: Cy – cytosol, A – apoplast, E – endoplasmic reticulum, Ch – chloroplast; controls: S – wild-type control grown on sucrose medium, G – wild-type control grown on glucose medium, D – DsRed expressing PCPs (positive control). Number above the blot are provided for easier orientation.

The *Trametes hirsuta* laccase accumulated to the highest levels among the five proteins we tested when targeted to the chloroplast (19.3 ± 4.8 mg kg^{-1}; ±SD, n = 3) or cytosol (14.5 ± 4.4 mg kg^{-1}, ±SD, n = 3) (Fig. 1A). Similarly, the highest accumulation of alkane monooxygenase (up to 8.3 ± 0.9 mg kg^{-1}, ±SD, n = 3) and ethyl acetate hydrolase (up to 5.3 ± 2.0 mg kg^{-1}, ±SD, n = 3), both from *P. putida*, was also observed in the chloroplast and cytosol (Fig. 1). The same trend was observed when expressing the B1 domain of protein G [80], polyphosphate kinases or biofilm degrading enzymes, which do not require complex post-translational modifications in BY-2 PCPs and whole plants [81] and for a GFP–hFGF21 fusion protein that accumulated to ~2 g kg^{-1} wet biomass in chloroplasts [82]. However, many of these studies do not benchmark their results against other compartments, preventing the meaningful and structured comparison of data. Furthermore, the His_6 tag we used for product detection can affect the compartment-dependent accumulation of the proteins, as reported for ferritin [24], underlining the need to screen for the optimal subcellular localization on a case-by-case basis.

4. Conclusion

We have shown that different classes of enzymes with the potential to convert odorous compounds in plant biomass and its derivatives can be produced within plant cells. This will facilitate the simultaneous expression of these enzymes along with a primary product (e.g., antibody or vaccine candidate), reducing the odor of the residual biomass once the product has been extracted. BY-2 cell-based expression was used for screening purposes in this study. In the future, the most suitable enzymes should be expressed in transgenic plants that are cultivated at large scale so that sufficient biomass quantities are available for secondary use like plant fiber-based insulation material. In such a setting, inducible promoter systems using chemical (e.g. ethanol) or environmental triggers (e.g. drought) can be useful to avoid negative impact on plant growth and primary product formation [36,83–85].

The next steps in the development of such plants will include (1) defined activity testing of the enzymes using reference substrates (2) impact analysis in a prototype setting (e.g., quantitation of odorous

substances in residual plant biomass expressing the enzymes), and (3) synergy assessment, in which combinations of enzymes are tested to suppress the most unpleasant smells. To do so, the specific composition of odorous substances should be determined for a relevant plant waste stream. In the mid-term, we should also evaluate strategies to immobilize the enzymes in the biomass to prevent loss during the aqueous extraction of a primary product. For example, lipid-based membrane anchors or cellulose binding domains derived from plant pathogens can be tested in this context [86–89].

Ethics approval

Not applicable.

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Code availability (software application or custom code)

Not applicable.

Consent for publication

All authors have seen a draft version of the manuscript and concur with its submission to the journal.

Declaration of competing interest

The authors have no conflict of interest to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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