Title	Enhancement of production of eugenol and its glycosides in transgenic aspen plants via genetic engineering.
Author(s)	Koeduka, Takao; Suzuki, Shiro; Iijima, Yoko; Ohnishi, Toshiyuki; Suzuki, Hideyuki; Watanabe, Bunta; Shibata, Daisuke; Umezawa, Toshiaki; Pichersky, Eran; Hiratake, Jun
Citation	Biochemical and biophysical research communications (2013), 436(1): 73-78
Issue Date	2013-06-21
URL	http://hdl.handle.net/2433/175512
Right	© 2013 Elsevier Inc.; 許諾条件により本文は2013-11-01に公開.
Type	Journal Article
Textversion	author

Title:

Enhancement of production of eugenol and its glycosides in transgenic aspen plants *via* genetic engineering

Authors:

Takao Koeduka[a,1,*], Shiro Suzuki[b,1], Yoko Iijima[c], Toshiyuki Ohnishi[d], Hideyuki Suzuki[e], Bunta Watanabe[a], Daisuke Shibata[e], Toshiaki Umezawa[b], Eran Pichersky[f], Jun Hiratake[a]

Affiliation:

[a]Institute for Chemical Research, Kyoto University, Uji, Kyoto, 611-0011, Japan

[b]Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Kyoto 611-0011, Japan

[c]Faculty of Applied Bioscience, Kanagawa Institute of Technology, 1030 Shimo-ogino, Atsugi, Kanagawa 243-0292, Japan

[d]Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

[e]Kazusa DNA Research Institute, 2-6-7 Kazusakamatari, Kisarazu, Chiba 292-0818, Japan

[f]Department of Molecular, Cellular and Developmental Biology, University of Michigan, 830 North University Street, Ann Arbor, MI 48109-1048, USA

[1]These authors contributed equally to this work.

[*]To whom correspondence should be addressed:

Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan
Tel: +81 774 38 3230

Fax: +81 774 38 3229
E-mail: takaori@scl.kyoto-u.ac.jp

Abstract

Eugenol, a volatile phenylpropene found in many plant species, exhibits antibacterial and acaricidal activities. This study attempted to modify the production of eugenol and its glycosides by introducing petunia *coniferyl alcohol acetyltransferase* (*PhCFAT*) and *eugenol synthase* (*PhEGS*) into hybrid aspen. Gas chromatography analyses revealed that wild-type hybrid aspen produced small amount of eugenol in leaves. The heterologous overexpression of *PhCFAT* alone resulted in up to 7-fold higher eugenol levels and up to 22-fold eugenol glycoside levels in leaves of transgenic aspen plants. The overexpression of *PhEGS* alone resulted in a subtle increase in either eugenol or eugenol glycosides, and the overexpression of both *PhCFAT* and *PhEGS* resulted in significant increases in the levels of both eugenol and eugenol glycosides which were nonetheless lower than the increases seen with overexpression of *PhCFAT* alone. On the other hand, overexpression of *PhCFAT* in transgenic *Arabidopsis* and tobacco did not cause any synthesis of eugenol. These results indicate that aspen leaves, but not *Arabidopsis* and tobacco leaves, have a partially active pathway to eugenol that is limited by the level of CFAT activity and thus the flux of this pathway can be increased by the introduction of a single heterologous gene.

Keywords

transgenic aspen; phenylpropene; monolignol; eugenol glycoside; metabolic engineering

Introduction

Eugenol belongs to the class of volatile phenylpropenes that are widely distributed across the plant kingdom. Many phenylpropenes impart a characteristic aroma, and some fresh fruits and processed products that contain them are widely consumed. For example, in the important vegetable crop tomato, many phenylpropene volatiles, including eugenol, are often stored as glycosides, representing an aroma reserve, and overall content of releasable phenylpropenes is an important factor determining the commercial value of the tomato fruits [1,2]. Volatile phenylpropenes also exhibit various physiological activities, antimicrobial and acaricidal activities [3,4], thus these compounds function as direct defense against microorganisms and herbivorous pests.

Eugenol is biosynthesized *via* two enzymatic steps. Coniferyl alcohol acetyltransferase (CFAT) catalyzes the first step in eugenol biosynthesis that branches off from the lignin pathway, and eugenol synthase (EGS) is the second and last enzyme responsible for synthesis of eugenol (Fig. 1). The two genes have been isolated and functionally characterized in basil (*Ocimum basilicum*), petunia (*Petunia hybrida* and *Petunia axillaris*), *Clarkia breweri*, and anise (*Pimpinella anisum*) [5-10].

To date, genetic modification of phenylpropene biosynthesis has been used to alter the volatile composition only in strawberry fruits [11]. In transgenic strawberry, the modification of anthocyanin biosynthesis by simultaneously expressing *EGS* and downregulating *chalcone synthase* (*CHS*) gene boosted the formation of eugenol, isoeugenol, and their analogs chavicol and anol (the latter two are phenylpropenes without a functional group at 3′ position of the aromatic ring, see Fig. 1), indicating that diverting the flavonoid pathway to phenylpropene biosynthesis through metabolic engineering [11] is also possible. Surprisingly, there is no report about the metabolic engineering in vegetative parts, using model plants such as tobacco and *Arabidopsis*. Hybrid aspen (*Populus tremula* × *tremuloides*), which is a relatively amenable tree species for transformation, has been utilized to genetically engineer the lignin biosynthetic pathway. Because the lignin pathway shares some of its precursors with the phenylpropene pathway (Fig. 1) and enhancing the productivity of eugenol *in planta* may contribute to improve plant protection, plants with high levels of lignin biosynthesis are attractive targets for genetic manipulation of the phenylpropene pathway. In this study, we attempted to engineer eugenol synthesis in tobacco, *Arabidopsis*, and hybrid aspen by introducing petunia *CFAT* and/or petunia *EGS* genes.

Materials and methods

Preparation and growth of transgenic plants

In preparation of transgenic hybrid aspen, tobacco and *Arabidopsis*, we first subcloned the coding sequences of *P. hybrida CFAT* (*PhCFAT*; GenBank accession ABG75942) and *P. hybrida EGS* (*PhEGS*; GenBank accession ABR24115) individually into the pENTR/D-TOPO vector (Life Technologies, Carlsbad, CA). The resulting entry vector was then recombinated with the destination vector pH35GS [12] or pMDC32 [13] using LR clonase II (Life Technologies). *Agrobacterium*-mediated transformation and regeneration of hybrid aspen (*Populus tremula* × *tremuloides* T89) were carried out as previously described [12] using the equivalent mixture of cells [14] of *A. tumefaciens* GV3101::pMP90 harboring pH35GS-PhCFAT and pH35GS-PhEGS (Supplementary Fig. 1). The regenerated transformants were screened by RT-PCR with the specific primers described as below. PCR-positive transgenic aspen were acclimatized in a growth chamber at 25˚C under a 16-h light/8-h dark photoperiod for one month. The plants were then transplanted into 1/10,000a Wagner pots containing a equivalent mixture of garden soil “Hanasaki Monogatari” (Akimoto-tensanbutsu, Iga, Japan) and vermiculite and were grown in a greenhouse at 25±2°C from June to October. The transgenic *Arabidopsis* and tobacco plants were produced by using *A. tumefaciens* strain AGL1 harboring pMDC32-PhCFAT (Supplementary Fig. 1) and a floral dip or a leaf disc protocol [15,16]. The obtained transformants were grown in the growth chamber at 22˚C for *Arabidopsis* and 25˚C for tobacco under a 14-h light/10-h dark photoperiod.

RT-PCR analysis

Total RNA was isolated from each leaf tissue with an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). The RNA was subjected to DNase treatment using the DNA-free kit

(Life Technologies), and the first strand cDNAs were synthesized using Superscript III reverse transcriptase (Life Technologies) with Oligo-dT primer. Semi-quantitative RT-PCR experiments with KOD FX Neo polymerase (Toyobo, Tokyo, Japan) were carried out for 32 cycles (98°C for 2 min and then cycling at 98°C for 15 sec, 54°C for 30 sec and 68°C for 30 sec). PCR primers for *PhCFAT* amplification were forward primer, 5′-TATTGACGATTCTAAAAGATGCAAACTT-3′, and reverse primer, 5′-TATTGACGATTCTAAAAGATGCAAACTT-3′. Primers (forward and reverse, respectively) for *PhEGS* were 5′-CCAACTTTGGTTCAGCCAGGAGC-3′ and 5′-TCCGCTCGAGTTAGGCAAAGTGACTAAGGTACTC-3′. Primers (forward and reverse, respectively) for *Actin*, which was used as reference for standardization of cDNA amounts, were 5′-GCCAGTGGTCGTACAACTGGTATTG-3′ and 5′-CCTTGATCTTCATGCTTGGAGC-3′.

Analysis of phenylpropene volatiles

Leaves (250 mg fresh weight) from each plant were ground using mortar and pestle in liquid nitrogen. The ground powder was homogenized and immersed with 2.0 mL of hexane containing guaiacol (20 μg) as an internal standard. The mixture was incubated at 25°C for 1 hr to extract eugenol. The extracts were dried over anhydrous sodium sulfate and concentrated to 100 μL by passing air-flow. The concentrated extracts were passed through a CENTRICUT W-MR column (MF 0.4 μm, Kurabo, Osaka, Japan) to remove any residue. Eugenol and other phenylpropene volatiles were analyzed by a Shimadzu GC-2014 or GCMS-QP2010 Plus equipped with a DB-5 column (15 m × 0.25 mm I.D. × 0.4 μm film thickness, Agilent Techonologies) as previously described [8]. Separation conditions were as follows: 80°C for the initial temperature using a 2-min hold, and then a temperature gradient from 80°C to 240 °C at 10°C min^{-1} was applied, followed by a 5-min hold at 240°C. Injection and detector temperatures were set at 250°C and 280°C, respectively. Eluted compounds were identified by comparing their retention time and mass fragmentation patterns with authentic standards.

LC/MS/MS analysis of eugenol glycosides in transgenic hybrid aspen

Each frozen leaf sample was powdered with a mortar and pestle. Powdered samples (100 mg) were extracted with 300 μL of extraction solvent (methanol containing 10 μg/mL genistein as an internal standard). After samples had been homogenized twice with a Mill MM 300 mixer (Qiagen, Hilden, Germany) at 27 Hz for 2 min, homogenates were centrifuged. The supernatant was removed, and an additional 300 μL was added to the residue, and the extraction was repeated. The pooled extracts were diluted with methanol to 10 times volume, and filtered through a 0.2 μm PVDF membrane (Whatman, Brentford, U.K.). The filtrate (10 μL) was applied to LC-MS.

A Finnigan LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA) coupled with an Agilent 1200 system (Agilent Technologies, Palo Alto, CA) was used for LC-MS analysis. A TSK-gel column ODS-100V (5 μm, 4.6 mm I.D. × 250 mm, TOSOH, Tokyo, Japan) was used for separation of eugenol glycosides by mobile phase consisting of 0.1% (v/v) aqueous formic acid (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B). The gradient program was as follows: 3 to 50% B for the first 20 min, 50 to 90% B from 20 to 40 min, 90% B from 40 to 45 min, and 95% B from 45 to 50 min, with a flow rate of 0.5 mL min^{-1}. The column oven temperature was set at 40°C. The MS system was operated in the positive mode according to a previously described method [17].

Quantification of eugenol glycosides in the leaves of hybrid aspen was performed using calibration curves prepared with eugenyl primeveroside.

*Purification of eugenyl 6-*O*-β-*D*-xylopyranosyl-β-*D*-glucopyranoside (eugenyl primeveroside)*

For purification of eugenyl 6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside (eugenyl primeveroside), the fresh leaves of *Camellia sasanqua* were plucked at the Center for Education and Research in Field Sciences, Shizuoka University (Fujieda, Shizuoka, Japan) in March from 2009 to 2011. Fresh young leaves were finely chopped and crushed in liquid nitrogen by a homogenizer. The fine powder was extracted three times with 80% methanol and filtered. The combined extracts were concentrated *in vacuo* and separated three times with hexane and H_2O. The combined aqueous fractions were applied on a solid phase extract column (Oasis HLB 3 cc Vac Cartridge, Waters, Milford, MA). The column was washed with H_2O and eluted with MeOH. The eluent was applied to a reversed-phase HPLC column (Cosmosil $5C_{18}$-MS-II, 4.6 mm I.D. × 150 mm, Nacalai Tesque, Kyoto, Japan) with gradient elution of 15 to 70% acetonitrile in H_2O and detection at UV 278 nm. The collected fractions were checked by HPLC-LC/MS (*m/z* 371, negative mode), and the fractions containing eugenyl primeveroside were further purified by HPLC (CAPCELL PAK C_{18} UG120, 4.6 mm I.D. × 250 mm, Shiseido, Tokyo, Japan) to afford the target compound. The molecular formula and ^1H and ^{13}C NMR spectroscopic data of the isolated compound was identical to eugenyl primeveroside as previously described [16].

Results

Generation of transgenic plants

Previous studies showed that two enzymes, CFAT and EGS, were required to convert coniferyl alcohol to eugenol [5,7]. Because detectable levels of coniferyl alcohol were observed in aspen differentiating xylem [19], we rationalized that the overexpression of CFAT and EGS would result in an increase in the production of eugenol in aspen. Therefore, we used a mixture of two different *Agrobacterium* transformants [14] harboring pH35GS-PhCFAT and pH35GS-PhEGS (Supplementary Fig. 1) to infect hybrid aspen stem segments. We obtained more than 50 transgenic lines after repeated subcultures containing hygromycin as a selective reagent. The transgene expression was checked by RT-PCR using leaves of the regenerated transformants (Fig. 2). Of these lines, *PhCFAT* alone was expressed in 18 lines, *PhEGS* alone was expressed in 26 lines, and both *PhCFAT* and *PhEGS* were expressed in 12 lines. All transgenic lines established with the *PhCFAT* and/or *PhEGS* genes showed the same morphological phenotype as wild-type plants (Supplementary Fig. 2). Some of the representative transformants that survived after transplanting in pots were used for the subsequent analysis.

Production of eugenol and its glycosides in transgenic aspen plants overexpressing PhCFAT and/or PhEGS

Leaves of transgenic lines and wild type of hybrid aspen were extracted with hexane and analyzed by GC-MS for the production of eugenol and other volatile compounds. GC analyses of *PhEGS*-overexpressing lines showed only a slight increase in eugenol level in hybrid aspen as compared to that of the wild type (the eugenol content of wild-type plants ranged from 6.2 to 8.8 μg/g fresh weight, with an average of 7.4 ± 1.3 μg/g fresh weight, whereas for *PhEGS*-expressing lines, an average of 11.3 ± 3.2 μg/g fresh weight). However, in the transgenic lines overexpressing both *PhCFAT* and *PhEGS* or only *PhCFAT*, a significant increase in the eugenol production was observed, with eugenol content measured at 52.5 and 38.9 μg/g fresh weight on average, respectively. In particular, the *PhCFAT* transformant no. 4 accumulated 85.1 μg eugenol/g fresh weight, which was 12-fold greater than that of the wild type (Fig. 3). It should be noted that no other phenylpropenes were detected in addition to eugenol in the transgenic lines (data not shown).

Eugenol is often detected as a glycosylated form in plants [1,20,21]. Therefore, the accumulation of eugenol glycosides in the leaves was analyzed using liquid chromatography/mass/mass spectrometry (LC/MS/MS) (Supplementary Fig. 3). When compared with the mass pattern and retention time of the standard eugenyl primeveroside, glycosylated eugenol was detected as a monoglycoside and a diglycoside in all of the transformant as well as the wild-type aspen plants. In the *PhCFAT*-overexpressing line, the maximal increase in both eugenol monoglycoside and diglycoside of 22 and 3.5-fold, respectively, was observed over the levels seen in wild-type plants, and the *PhCFAT* and *PhEGS* double transformants exhibited 7.5 and 2.5-fold increases over wild type (Fig. 4). In contrast, the level of eugenol glycosides from *PhEGS* single transformants was only slightly higher than the wild-type level. A similar accumulation pattern between the level of eugenol glycosides and its aglycone was observed among all the transformants (Fig. 3, 4).

Since transgenic aspen leaves expressing only *PhCFAT* showed a significant increase in eugenol biosynthesis, we checked to see if aspen leaves already express an endogenous gene encoding EGS. This was a reasonable hypothesis particularly in light of the observations that non-transgenic aspen leaves already synthesize low levels of eugenol (Fig. 3). A search of the database (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=poplar) identified several aspen cDNAs that encode proteins with high identity to *bona fide* EGS proteins (Table 1). Moreover, transcripts of some of these genes were identified in leaf RNA (Table 1).

As expressing *PhCFAT* by itself led to a large increase in eugenol production in hybrid aspen, we next examined whether transforming tobacco and *Arabidopsis* plants with *PhCFAT* would also result in increased eugenol production. The T2 generation plants of transformants with pMDC32-PhCFAT (Fig. 2) were analyzed, and, in contrast to hybrid aspen, no significant increase in the eugenol production was observed in both transgenic tobacco and *Arabidopsis* harboring *PhCFAT* as compared to the wild type (Supplementary Table 1).

Discussion

Recently, Hoffman et al [11] reported that the expression of basil *EGS* gene or petunia

IGS (*isoeugenol synthase*) in strawberry fruit, which is rich in anthocyanins (pigments that are derived from *p*-coumaroyl CoA), led to a large increase in the levels of eugenol, chavicol, isoeugenol, and anol. The presence of phenylpropene glycosides was not examined. Based on these results, they concluded that the strawberry fruit already expresses the putative acyl transferases that produce monolignol acetates but lacks the final enzyme (EGS or IGS) for phenylpropene biosynthesis. This conclusion was supported by the observation that *p*-coumaryl acetate accumulated in the transgenic strawberry fruits expressing a *CHS*-RNAi construct (suppressing chalcone synthase).

In our investigation in aspen we have observed that expressing *PhCFAT* by itself greatly increased the production of eugenol, and expressing *PhEGS* in addition to *PhCFAT* also caused an increase in eugenol production, but at a lower level compared with *PhCFAT* alone, while expressing *PhEGS* by itself resulted in only a minor increase in eugenol levels over those seen in wild-type aspen plants. These observations suggest that, as in strawberries, aspen has a latent ability to biosynthesize eugenol, but that the limiting enzyme in aspen is not EGS but CFAT. Thus, the introduction of *PhCFAT* to aspen plants led to a much higher flux for monolignol acetates and hence a significant increase in eugenol biosynthesis. This conclusion is consistent with the observations that (1) wild-type aspen leaves contain some eugenol, (2) their vascular tissue (including the prominent veins in the leaves) is known to synthesize monolignols, and (3) genes with significant sequence identity to *bona fide* EGS enzymes have been found to be expressed in aspen leaf tissue.

The expression of *PhCFAT* did not lead to the production of other phenylpropenes such as chavicol and methoxyeugenol in the transgenic aspen leaves (data not shown). One possible explanation for this observation is that, even though PhCFAT was present in the transgenic plants, the PhCFAT activities against *p*-coumaryl alcohol and sinapyl alcohol is lower than its activity with coniferyl alcohol, as previously reported [7], or that the levels of *p*-coumaryl alcohol and sinapyl alcohol were not sufficient for chavicol or methoxyeugenol formation *in planta*, suggesting that the phenylpropene production is dependent on *in vivo* production of monolignols and their acetates.

In addition to the increase in eugenol levels, *PhCFAT* overexpression with/without *PhEGS* expression also led to a proportional increase in the levels of eugenol glycosides in transgenic hybrid aspen (Fig. 4). Because eugenol is generally toxic to plant cells as well as to herbivores, free eugenol is often stored in the compartmentalized tissue such as trichomes [22] or is converted to its glycosylated forms that can be stored in vacuoles. It was previously reported that tomato fruits and leaves of *Camellia sasanqua* contain glycosylated eugenol rather than its aglycone [1,20]. In this study, we used eugenyl 6-*O*-(β-D-xylopyranosyl)-β-D-glucopyranoside (eugenyl primeveroside) isolated from *C. sasanqua* leaves as a standard. However, the mass spectroscopic analysis indicated that the aspen eugenol diglycosides are not identical to that of *C. sasanqua* leaves although the MS/MS spectra are very similar [Supplementary Fig. 3 (E) and (F)].

In contrast to the situation in aspen, where small amounts of eugenol were already found in wild-type plants and the introduction of *PhCFAT* alone greatly increased these amounts, wild-type tobacco and *Arabidopsis* plants did not contain any eugenol, and the introduction of *PhCFAT* did not lead to any eugenol synthesis. This observation suggests that these plants are missing multiple enzymes in the pathway to phenylpropene in the leaf tissues examined.

This report demonstrates that the overexpression of *PhCFAT* in transgenic hybrid aspen leads to higher levels of eugenol and eugenol glycosides. For *in vivo* phenylpropene production, the supply of the endogenous monolignols and their acetates in the host plants is clearly a prerequisite in attempt to manipulate phenylpropene levels. In future work it would be of interest to investigate if the increased levels of eugenol in the transgenic plants correlates with antimicrobial and acaricidal activities.

Acknowledgement

We thank Dr. Kazufumi Yazaki of Kyoto University for his valuable advice and Ms. Naoko Tsue of Kyoto University for her technical assistance. We also acknowledge Dr. Taku Demura of Nara Institute of Science and Technology, Dr. Csaba Koncz of Max-Planck Institute for Plant Breeding Research, and Dr. Björn Sundberg of Umeå Plant Science Centre for providing pH35GS vector, *A. tumefaciens* GV3101::pMP90, and *P. tremula* × *tremuloides* T89, respectively. We also thank DASH (Development and Assessment of Sustainable Humanosphere) system of Research Institute for Sustainable Humanosphere, Kyoto University. This study was supported in part by the Shorai Foundation for Science and Technology.

References

[1] Y.M. Tikunov, R.C. de Vos, A.M. González Paramás, R.D. Hall, A.G. Bovy, A role for differential glycoconjugation in the emission of phenylpropanoid volatiles from tomato fruit discovered using a metabolic data fusion approach, Plant Physiol. 152 (2010) 55-70.

[2] S. Birtić, C. Ginies, M. Causse, C.M. Renard, D. Page, Changes in volatiles and glycosides during fruit maturation of two contrasted tomato (*Solanum lycopersicum*) lines, J. Agric. Food Chem. 57 (2009) 591-598.

[3] P. Zhang, E. Zhang, M. Xiao, C. Chen, W. Xu, Enhanced chemical and biological activities of a newly biosynthesized eugenol glycoconjugate, eugenol α-D-glucopyranoside, Appl. Microbiol. Biotechnol. 97 (2013) 1043-1050.

[4] C. Pasay, K. Mounsey, G. Stevenson, R. Davis, L. Arlian, M. Morgan, D. Vyszenski-Moher, K. Andrews, J. McCarthy, Acaricidal activity of eugenol based compounds against scabies mites, PLoS One 5 (2010) e12079.

[5] T. Koeduka, E. Fridman, D.R. Gang, D.G. Vassão, B.L. Jackson, C.M. Kish, I. Orlova, S.M. Spassova, N.G. Lewis, J.P. Noel, T.J. Baiga, N. Dudareva, E. Pichersky, Eugenol and isoeugenol, characteristic aromatic constituents of spices, are biosynthesized via reduction of a coniferyl alcohol ester, Proc. Natl. Acad. Sci. USA 103 (2006) 10128-10133.

[6] G.V. Louie, T.J. Baiga, M.E. Bowman, T. Koeduka, J.H. Taylor, S.M. Spassova, E. Pichersky, J.P. Noel, Structure and reaction mechanism of basil eugenol synthase, PLoS One 2 (2007) e993.

[7] R. Dexter, A. Qualley, C.M. Kish, C.J. Ma, T. Koeduka, D.A. Nagegowda, N. Dudareva, E. Pichersky, D. Clark, Characterization of a petunia acetyltransferase involved in the biosynthesis of the floral volatile isoeugenol, Plant J. 49 (2007) 265-275.

[8] T. Koeduka, G.V. Louie, I. Orlova, C.M. Kish, M. Ibdah, C.G. Wilkerson, M.E. Bowman, T.J. Baiga, J.P. Noel, N. Dudareva, E. Pichersky, The multiple phenylpropene synthases in both Clarkia breweri and Petunia hybrida represent two distinct protein lineages, Plant J. 54 (2008) 362-374.

[9] T. Koeduka, T.J. Baiga, J.P. Noel, E. Pichersky, Biosynthesis of t-anethole in anise: characterization of t-anol/isoeugenol synthase and an O-methyltransferase specific for a C7-C8 propenyl side chain, Plant Physiol. 149 (2009) 384-394.

[10] T. Koeduka, I. Orlova, T.J. Baiga, J.P. Noel, N. Dudareva, E. Pichersky, The lack of floral synthesis and emission of isoeugenol in *Petunia axillaris* subsp. *parodii* is

due to a mutation in the isoeugenol synthase gene, Plant J. 60 (2009) 961-969.

[11] T. Hoffmann, R. Kurtzer, K. Skowranek, P. Kiessling, E. Fridman, E. Pichersky, W. Schwab, Metabolic engineering in strawberry fruit uncovers a dormant biosynthetic pathway, Metab. Eng. 13 (2011) 527-531.

[12] M. Kubo, M. Udagawa, N. Nishikubo, G. Horiguchi, M. Yamaguchi, J. Ito, T. Mimura, H. Fukuda, T. Demura, Transcription switches for protoxylem and metaxylem vessel formation. Genes Dev. 19 (2005) 1855-1860.

[13] M.D. Curtis, U. Grossniklaus, A gateway cloning vector set for high-throughput functional analysis of genes in planta, Plant Physiol. 133 (2003) 462-469.

[14] L. Li, Y. Zhou, X. Cheng, J. Sun, J.M. Marita, J. Ralph, V.L. Chiang, Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. Proc. Natl. Acad. Sci. USA 100 (2003) 4939-4944.

[15] A. Bent, *Arabidopsis thaliana* floral dip transformation method, Methods Mol. Biol. 343 (2006) 87-103.

[16] R.B. Horsch, J.E. Fry, N.L. Hoffmann, D. Eichholtz, S.G. Rogers, R.T. Fraley, A simple and general method for transferring genes into plants, Science, 227 (1985) 1229-1231.

[17] Y. Iijima, Y. Nakamura, Y. Ogata, K Tanaka, N. Sakurai, K. Suda, T. Suzuki, H. Suzuki, K. Okazaki, M. Kitayama, S. Kanaya, K. Aoki, D. Shibata, Metabolite annotations based on the integration of mass spectral information, Plant J. 54 (2008)

949-962

[18] M. Straubinger, H. Knapp, N. Watanabe, N. Oka, H. Washio, P. Winterhalter, Three novel eugenol glycosides from rose flowers, *Rosa damascena* Mill, Natural Product Letters 13 (1999) 5-10.

[19] S. Suzuki, N. Sakakibara, L. Li, T. Umezawa, V.L. Chiang, Profiling of phenylpropanoid monomers in developing xylem tissue of transgenic aspen (*Populus tremuloides*). J. Wood Sci. 56 (2010) 71-76.

[20] C.R. Parks, K. Kondo, T. Swain, Phytochemical evidence for the genetic contamination of *Camellia sasanqua* Thunberg, Japan J. Breed. 31 (1981) 168-182.

[21] S. Hammami, M.L. Ciavatta, H.B. Jannet, G. Cimino, Z. Mighri, Three phenolic and a sterol glycosides identified for the first time in *Matthiola longipetala* growing in Tunisia, Croatica Chemica Acta 79 (2006) 215-218.

[22] D.R. Gang, T. Beuerle, P. Ullmann, D. Werck-Reichhart, E. Pichersky, Differential production of meta hydroxylated phenylpropanoids in sweet basil peltate glandular trichomes and leaves is controlled by the activities of specific acyltransferases and hydroxylases, Plant Physiol. 130 (2002) 1536-1544.

Table legend

Table 1. Genes encoding EGS-like proteins in *Populus* EST databases.

Figure legend

Figure 1. Formation of phenylpropenes in transgenic plants.

Eugenol is formed with the subsequent reactions by coniferyl alcohol acetyltransferase (CFAT) and eugenol synthase (EGS) from coniferyl alcohol. Eugenol is glycosylated by the endogenous glycosyltransferase (GT). Lignins are produced by the polymerization of monolignols such as *p*-coumaryl alcohol and coniferyl alcohol. The minor pathway leading to hydroxyphenyl lignin was indicated by a broken arrow. Chalcone synthase (CHS) catalyzes the formation of flavonoids and anthocyanins. The phenylpropene pathway was indicated by a broken line.

Figure 2. Expression of *coniferyl alcohol acetyltransferase* and *eugenol synthase* in independent transformants.

Semi-quantitative polymerase chain reaction analysis confirmed *PhCFAT* and *PhEGS* transcript levels in transgenic hybrid aspen. Actin expression levels were used as an internal control. Expression of *PhCFAT* mRNAs in transgenic tobacco (SR1) and *Arabidopsis* (Col-0) were also shown. WT indicates wild type.

Figure 3. The accumulation of eugenol in transgenic hybrid aspen.

(A) Representative gas chromatograms of hexane extracts from aspen leaves.

(B) Level of eugenol accumulated in aspen leaves.

Figure 4. The accumulation of eugenol glycosides in transgenic hybrid aspen.

The amount of the glycosides is represented as relative abundance (Wild type = 1)

(A) Level of eugenol monoglycoside in aspen leaves.

(B) Level of eugenol diglycoside in aspen leaves.

Supplementary data

Supplementary Figure 1. The constructs for overexpressing of *PhCFAT* and *PhEGS* under control of the 35S promoter.

(A) The constructs used for overexpression of *PhCFAT* and *PhEGS* in hybrid aspen.

(B) The constructs used for overexpression of *PhCFAT* in tobacco and *Arabidopsis*.

Supplementary Figure 2. Morphology of transgenic plants overexpressing *PhCFAT* and/or *PhEGS*.

Supplementary Figure 3. LC/MS/MS analysis of eugenol glycosides produced by transgenic hybrid aspen overexpressing *PhCFAT*.

(A) HPLC chromatogram of eugenol monoglycoside extracted from *PhCFAT* transformants.

(B) HPLC chromatogram of eugenol diglycoside extracted from *PhCFAT* transformants.

(C) HPLC chromatogram of authentic eugenyl primeveroside.

The metabolic products and authentic eugenol glycoside were analyzed by LC/MS/MS using selected ion monitoring at *m/z* 371 for eugenol monoglycoside (D) and at *m/z* 503 for eugenol diglycoside (E) and authentic eugenyl primeveroside (F).

Supplementary Table 1. Production of eugenol in transgenic plants with overexpression of *PhCFAT*.

Table 1. Genes encoding EGS-like proteins in *Populus* EST databases.

Sequence ID	Annotation	Length (bp)	Identity to PhEGS (%)	Expressed in leaf tissue
TC142239	Isoflavone reductase-like	1991	76	yes
TC147936	Leucoanthocyanidin reductase-like	1492	41	yes
TC138212	Phenylcoumaran benzylic ether reductase-like	1321	77	yes
TC141798	Isoflavone reductase-like	1211	55	no
TC145463	Phenylcoumaran benzylic ether reductase-like	1178	77	no
TC144261	Phenylcoumaran benzylic ether reductase-like	1030	78	yes
TC148965	Pinoresinol-lariciresinol reductase-like	976	49	no
CK320793	Phenylcoumaran benzylic ether reductase-like	812	76	no
TC154756	Phenylcoumaran benzylic ether reductase-like	754	74	no

Table 1. Koeduka et al.

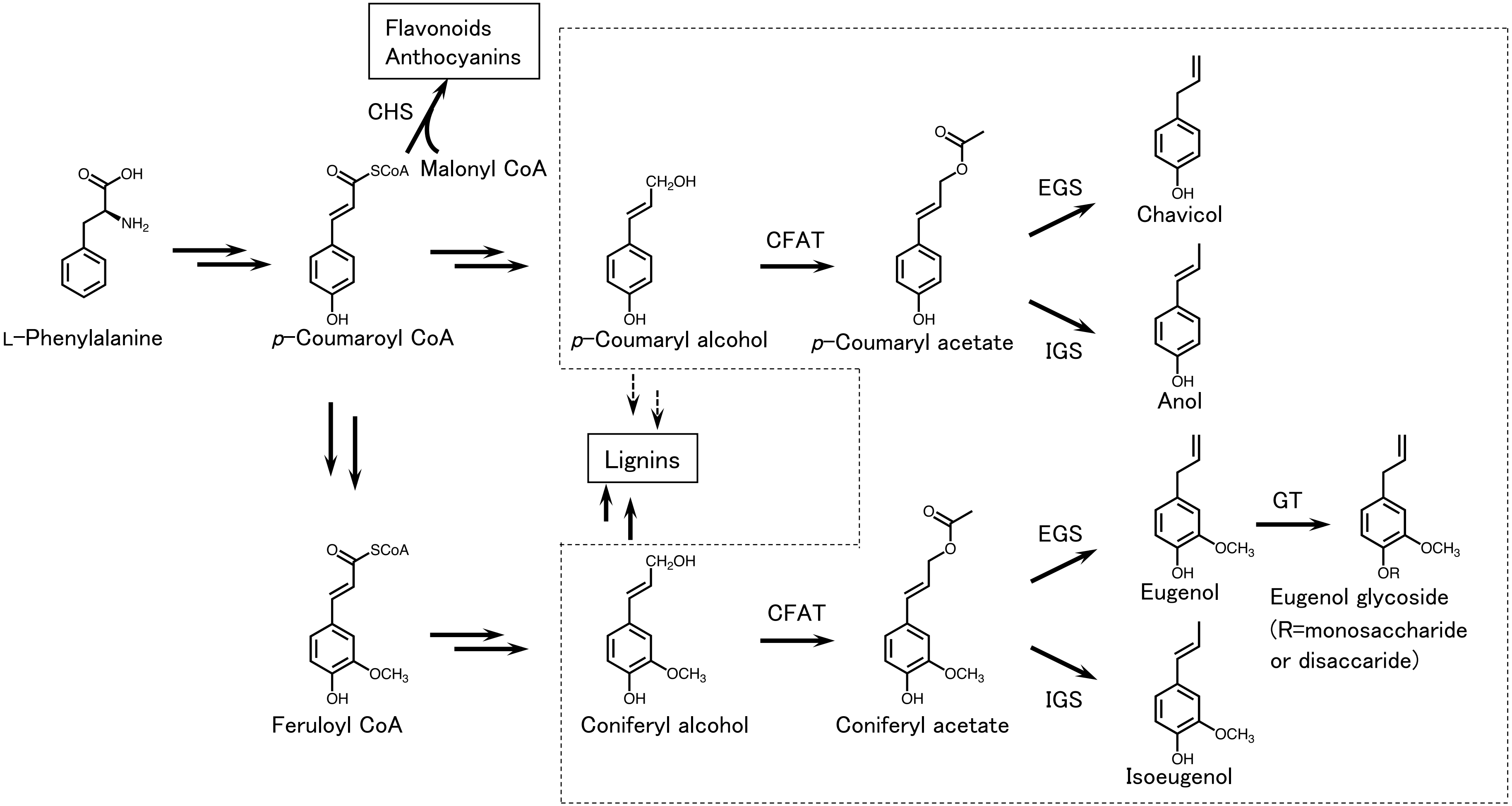


Figure 1. Koeduka et al.

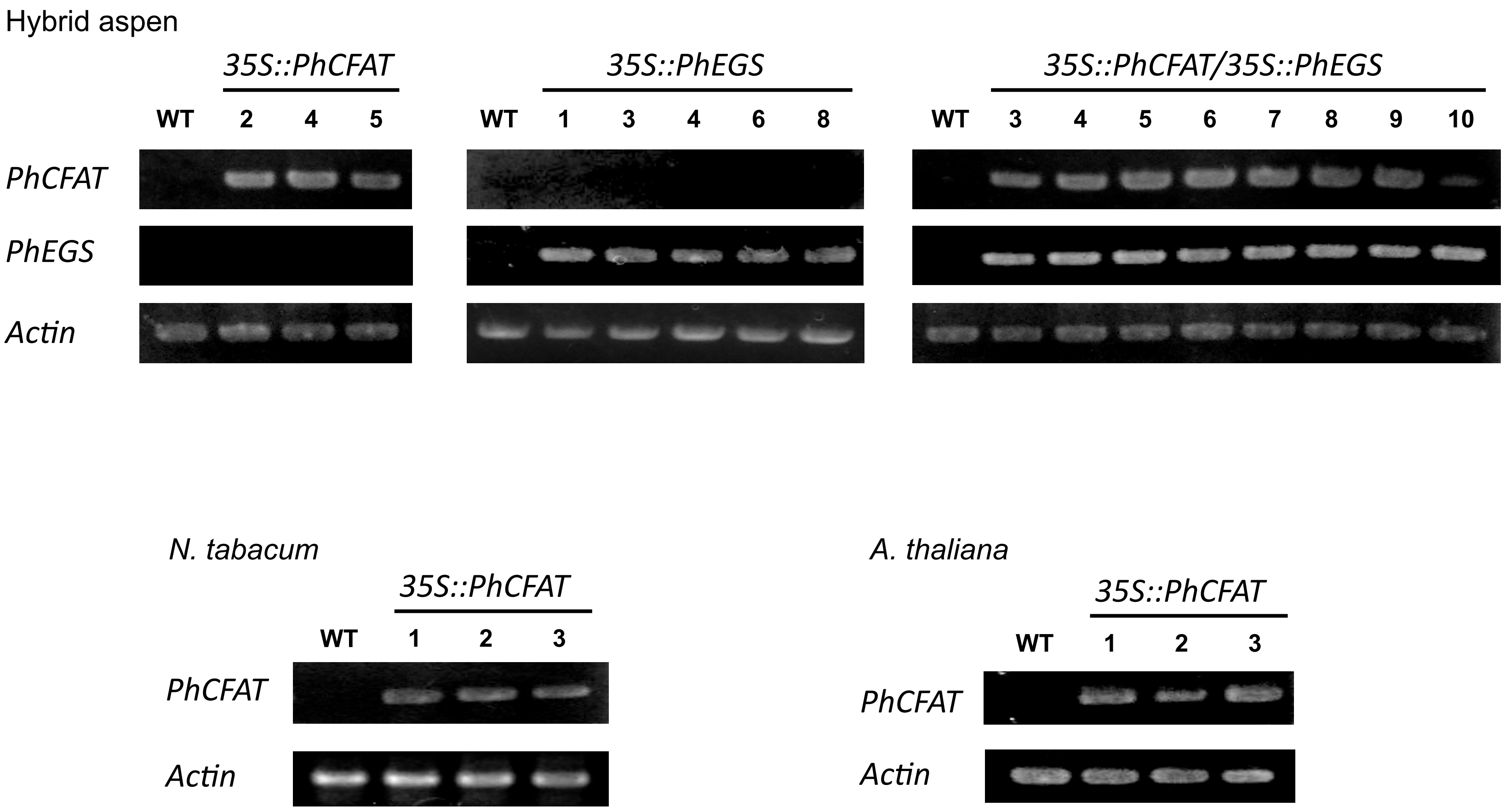


Figure 2. Koeduka et al.

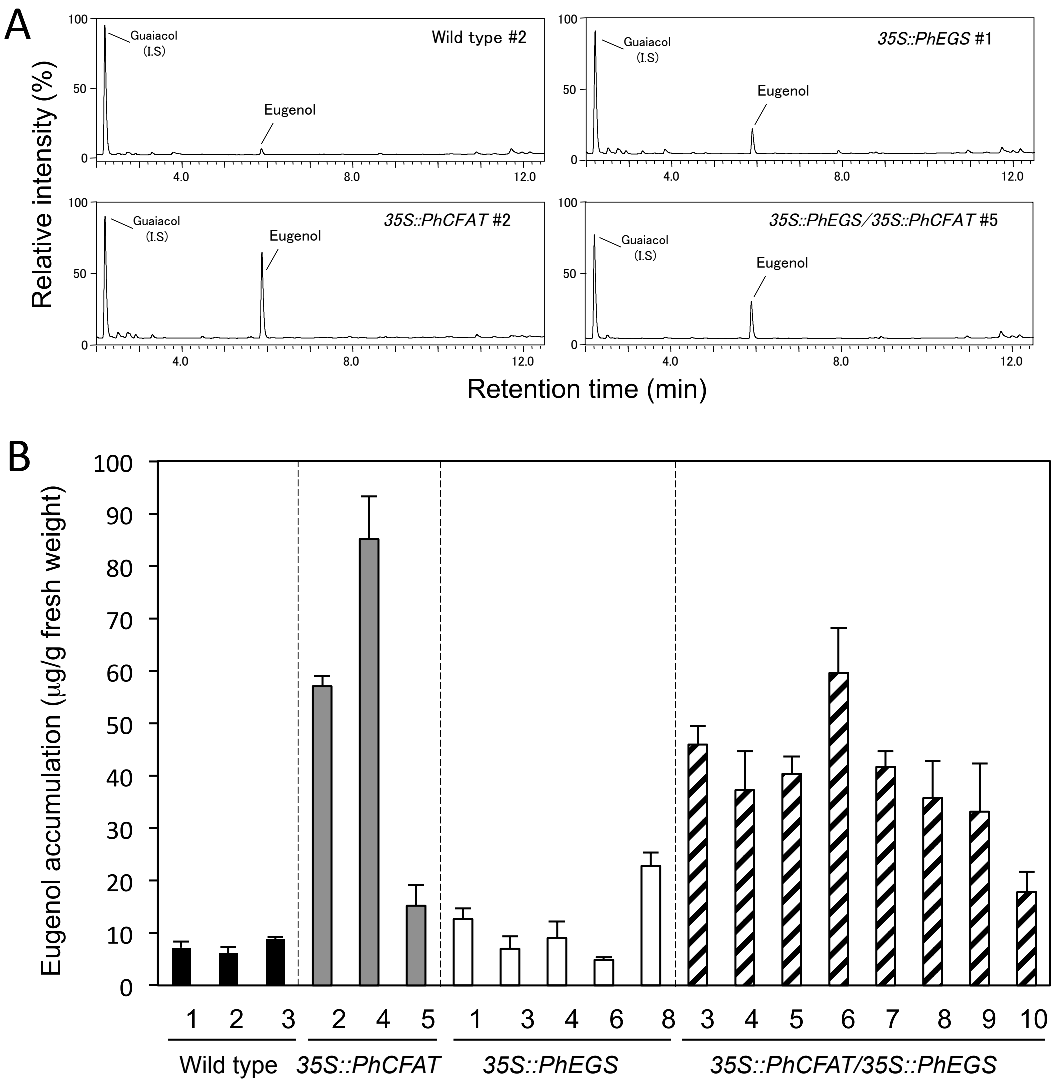


Figure 3. Koeduka et al.

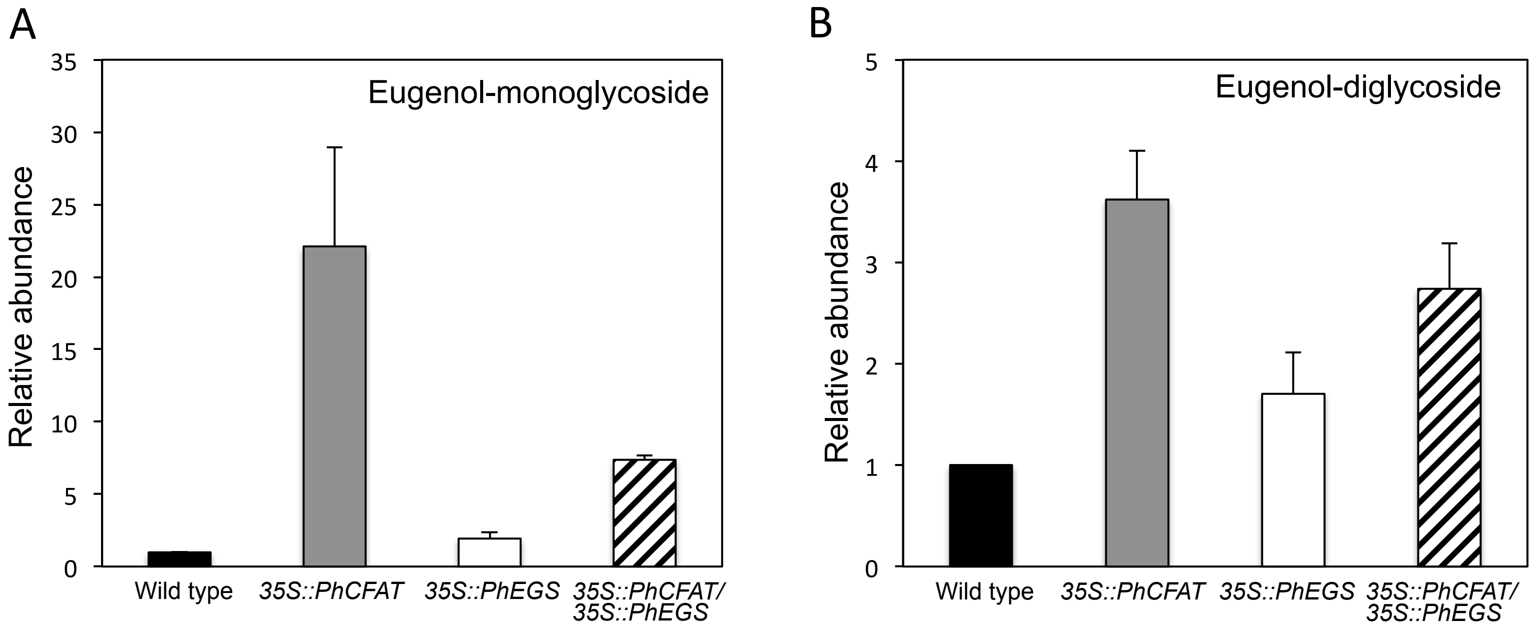
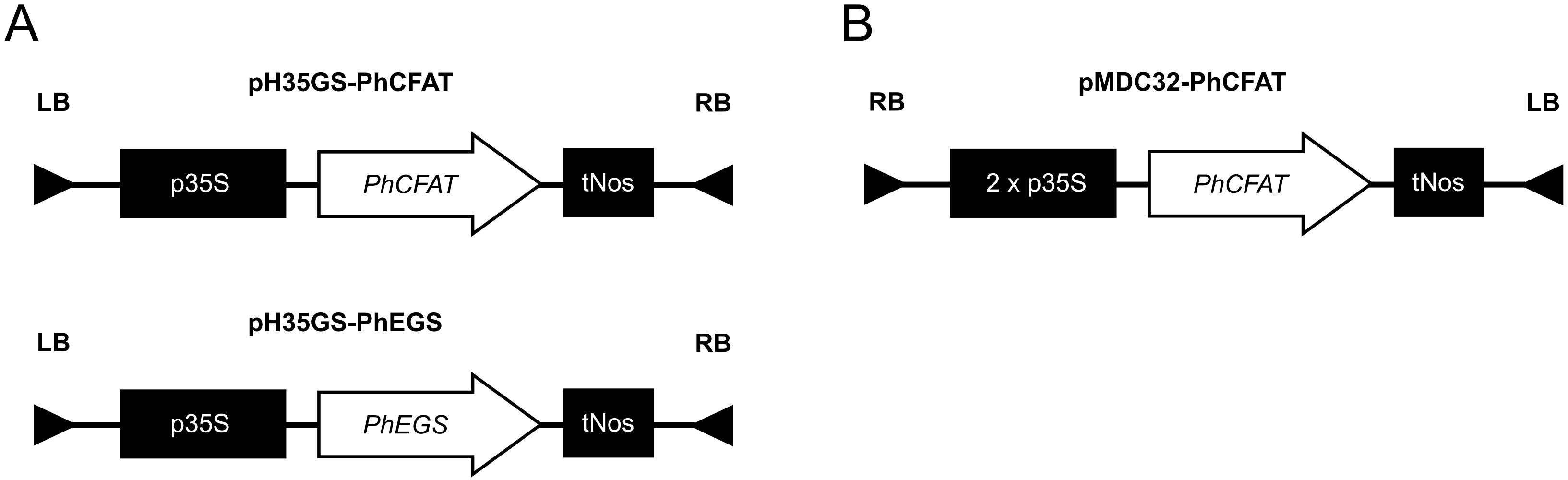
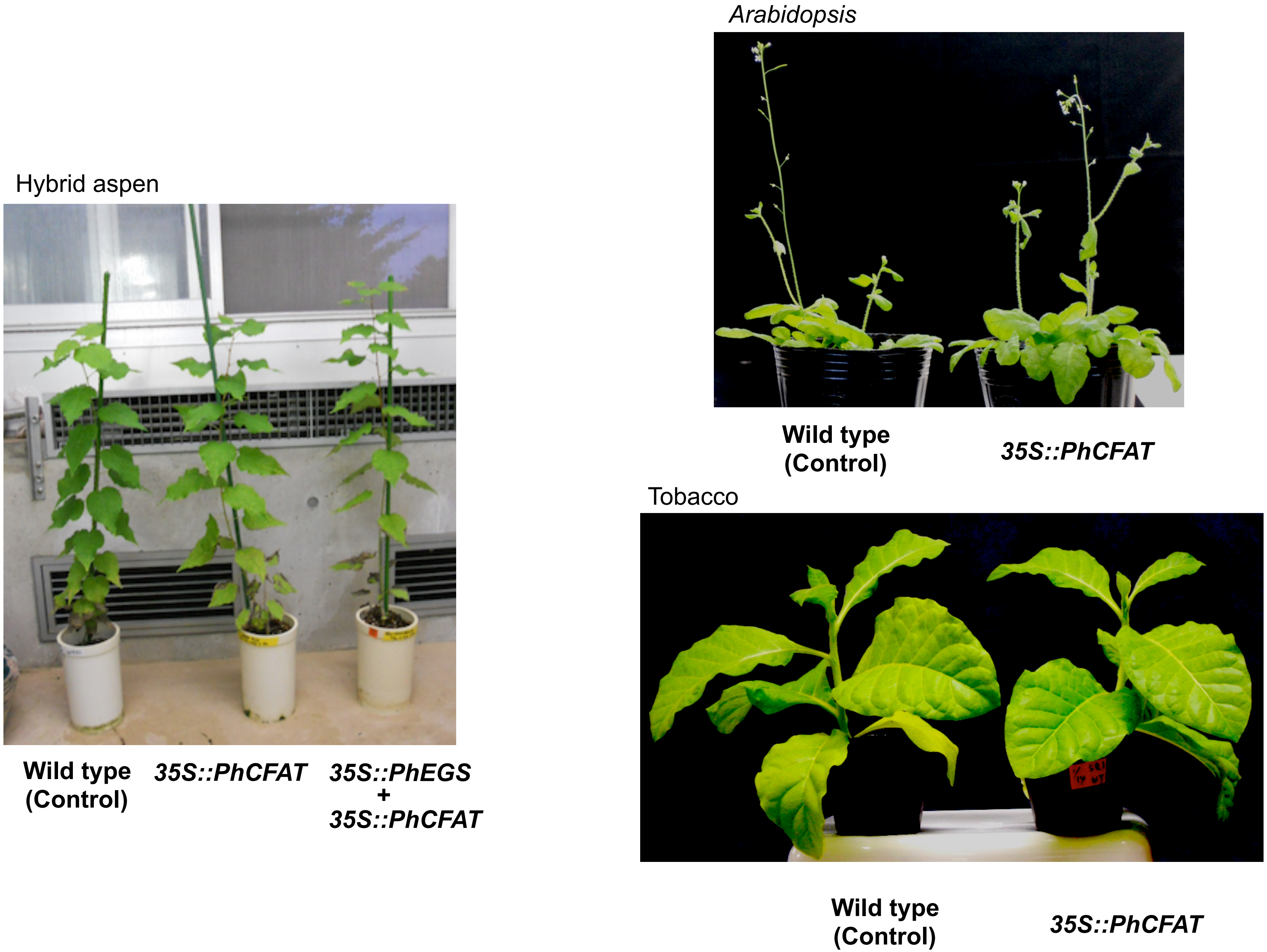


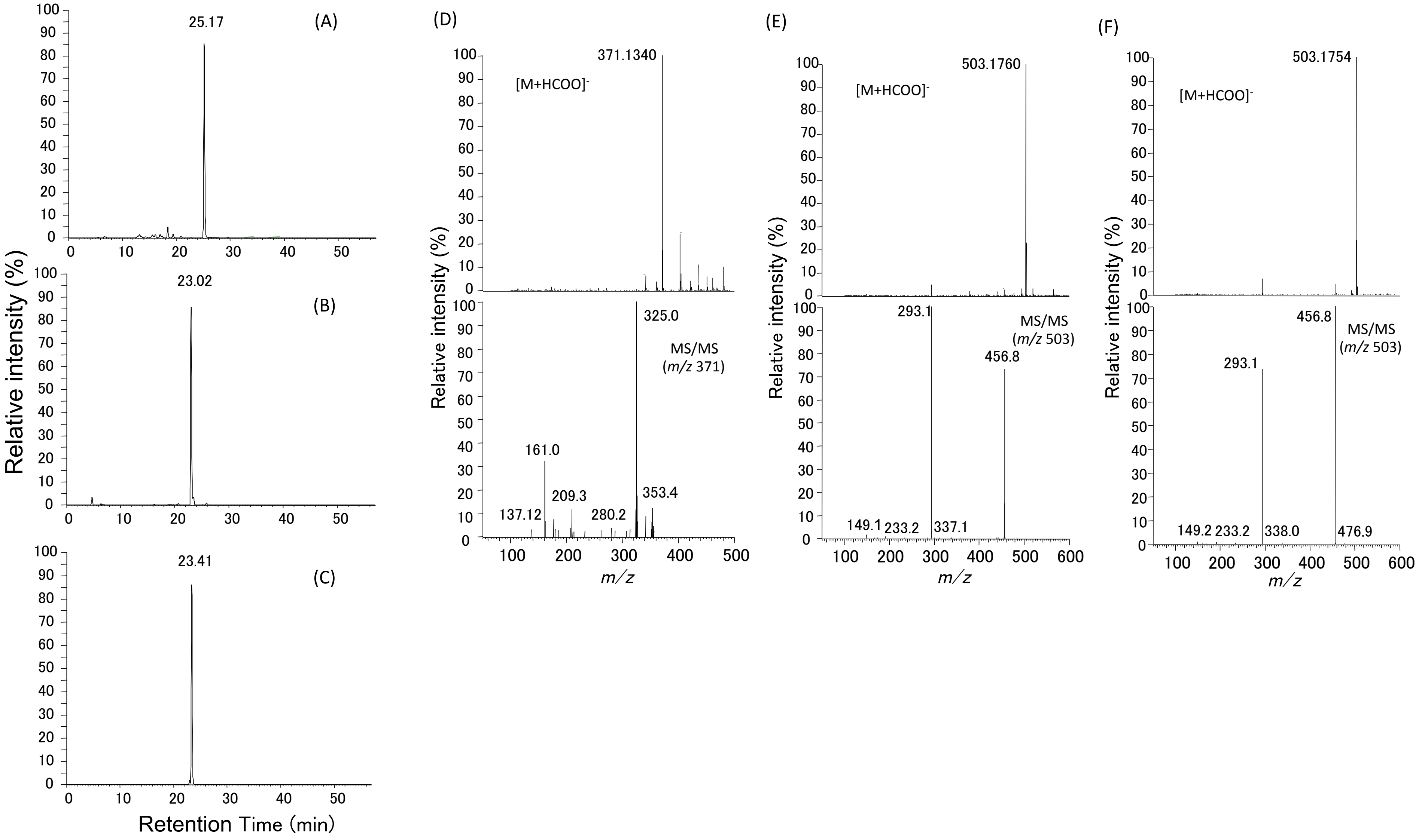
Figure 4. Koeduka et al.



Supplementary Fig. 1. Koeduka et al.



Supplementary Fig. 2. Koeduka et al.



Supplementary Fig. 3. Koeduka et al.

Supplementary Table 1. Production of eugenol in transgenic plants with overexpression of *PhCFAT*.

Host plants	Line	Produced eugenol
		μg/g fresh weight
Hybrid aspen	wild type	7.5 ± 1.3
	PhCFAT	52.4 ± 20.3
Tobacco	wild type	n.d.
	PhCFAT	n.d.
Arabidopsis	wild type	n.d.
	PhCFAT	n.d.

n.d., Not detectable

Supplementary Table 1. Koeduka et al.