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β-Amyrin synthase (EsBAS) and β-amyrin 28-oxidase (CYP716A244) in oleanane-type triterpene saponin biosynthesis in *Eleutherococcus senticosus*

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ABSTRACT

Siberian ginseng (*Eleutherococcus senticosus*) is a woody medical shrub belonging to the Araliaceae family. *E. senticosus* contains various types of saponins, including oleanane, noroleanane, lupane, and 3,4-secolupane types, depending on the aglycone structure. Oleanane-type triterpenes are the major saponin components in *E. senticosus*. Two enzymes (β-amyrin synthase and β-amyrin 28-oxidase) are essential for oleanane-type saponin biosynthesis from 2,3-oxidosqualene. In the present study, two full-length cDNAs encoding *EsBAS* and *CYP716A244* were isolated based on transcriptomics analysis of plant leaves. Both β-amyrin synthase (EsBAS) and β-amyrin 28-oxidase (CYP716A244), isolated from *E. senticosus*, were functionally characterised. β-amyrin production was confirmed by heterologous expression of the *EsBAS* gene in yeast and tobacco. Oleanolic acid production was confirmed by co-expression of both *EsBAS* and *CYP716A244* in engineered yeast and transgenic tobacco.

1. Introduction

Triterpenoid saponins are synthesised via the isoprenoid pathway. The first step in triterpenoid biosynthesis is the cyclisation of 2,3-oxidosqualene (**1**) (Fig. 1) by oxidosqualene cyclase (OSC) (Abe et al., 1993). The enzyme diversity of OSCs in plants leads to variations of triterpenoids (Xu et al., 2004). The triterpenoid backbone undergoes various modifications via hydroxylation or oxidation by cytochrome P450 (CYP) and glycosylation by uridine diphosphate glycosyltransferase (UGT). These CYP and UGT enzymes exist as supergene families in the plant genome.

Eleutherococcus senticosus Maxim (= *Acanthopanax senticosus*), also called Siberian ginseng, is a woody shrub belonging to the Araliaceae family. This plant is distributed in the Russian Far East, northeast China, Korea, and Japan and has been used as an important medicinal plant. The roots and stems of this plant are commonly used as a tonic and sedative and have also been used to treat rheumatism and diabetes (Umeyama et al., 1992; Davydov and Krikorian, 2000). The bioactive compounds of *E. senticosus* are saponins, isofraxidin, acanthosides, daucosterol and sesamin (Davydov and Krikorian, 2000). The saponins produced in *E. senticosus* are classified into oleanane, noroleanane, lupane, and 3,4-secolupane types based on the aglycone structure (Hwang et al., 2015). Oleanane-type triterpene saponins, such as eleutherosides I, K, L, and M (**5**), and ciwujianosides A1, A3, C3, C4, and D1, are the major saponins of *E. senticosus*.

The oleanane-type triterpene backbone is β-amyrin (**2**), a product of 2,3-oxidosqualene cyclase (Kushiro et al., 1998). β-Amyrin (**2**) is oxidised at the C-28 position in a sequential three-step oxidation by a single cytochrome P450 enzyme to yield oleanolic acid (**4**) through erythrodiol (**3**) (Fig. 1) (Carelli et al., 2011; Fukushima et al., 2011; Han et al., 2013). Both β-amyrin (**2**) and β-amyrin 28-oxidase play key roles in oleanane-type saponin biosynthesis in *E. senticosus*. The characterisation of these two genes is essential to understand their functional mechanisms in saponin biosynthesis and control the production of oleanane-type saponin via molecular breeding and metabolic engineering.

Previously, many candidate genes involved in saponin biosynthesis were isolated by transcriptomics analysis of *E. senticosus* (Hwang et al., 2015). In the present study, β-amyrin synthase

Abbreviations: CYP, cytochrome P450; OSC, oxidosqualene cyclase; GC-MS, gas chromatography–mass spectrometry; ORF, open reading frame; EsBAS, *Eleutherococcus senticosus* β-amyrin synthase; RT-PCR, reverse transcription-PCR; qPCR, quantitative real-time RT-PCR; UGT, glycosyltransferase; TIC, total ion chromatogram; LCMS-IT-TOF, liquid chromatograph mass spectrometer-ion trap/time-of-flight.

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(EsBAS) and β-amyrin 28-oxidase (CYP716A244) were characterised by heterologous expression of their cDNAs in yeast and produced β-amyrin (**2**) and oleanolic acid (**4**) in transgenic tobacco.

2. Results

2.1. Characterisation of the β-amyrin synthase (EsBAS) of E. senticosus

Full-length cDNA of *EsBAS1* was isolated from expressed sequence tags (ESTs) from the leaf cDNA library of *E. senticosus*. The *EsBAS* cDNA was 2738 bp long, including a 2295-bp open reading frame (ORF). The deduced amino acid sequence of EsBAS (769 amino acids with a predicted molecular mass of 88.4 kDa) is 93% identical to that of β-amyrin synthase in *Panax ginseng* (*PgPNY1*) and 80% identical to β-amyrin synthase in *Betula platyphylla* (OSCBPY). A phylogenetic tree (Fig. 2) constructed from the deduced amino acid sequences of plants indicated that EsBAS is grouped within β-amyrin synthases.

The transcription activities of *EsBAS* and *CYP716A244* were monitored via qPCR (Fig. 3). The transcripts of *EsBAS* were detected in all organs (leaf, stem bark and roots), although the expression levels were different, with the highest expression detected in the roots (Fig. 3a). The quantitative order of *EsBAS* mRNA accumulation was roots > stem barks > leaves. Methyl jasmonate (MeJA) treatment is an effective method to stimulate the biosynthesis of many secondary metabolites (Lambert et al., 2011). qPCR was performed to monitor the transcriptional levels of *EsBAS* at 0, 12, 24, 48, and 72 h after MeJA treatment. The transcription of *EsBAS* was clearly enhanced at 12 and 24 h after MeJA treatment and reduced to the level of the control (without MeJA treatment) at 72 h after MeJA treatment (Fig. 3b).

The ORF of *EsBAS* cDNA was expressed in ERG7-deficient mutant yeast (Karst and Lacroute, 1977). Gas chromatography mass spectrometry (GC/MS) indicated that the total ion chromatogram (TIC) of the yeast extracts showed a new peak at a

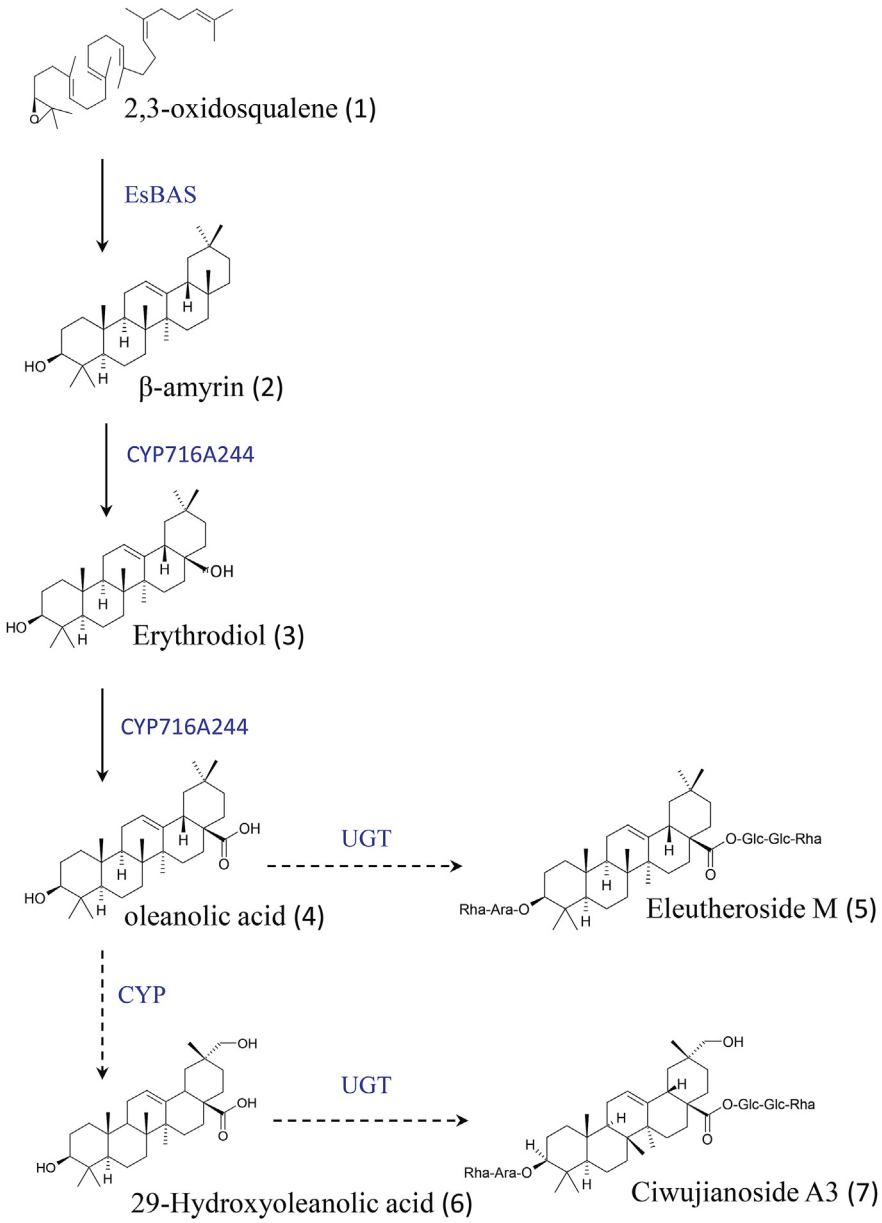

Fig. 1. Putative saponin biosynthetic pathway from 2,3-oxidosqualene (**1**) in *E. senticosus*.

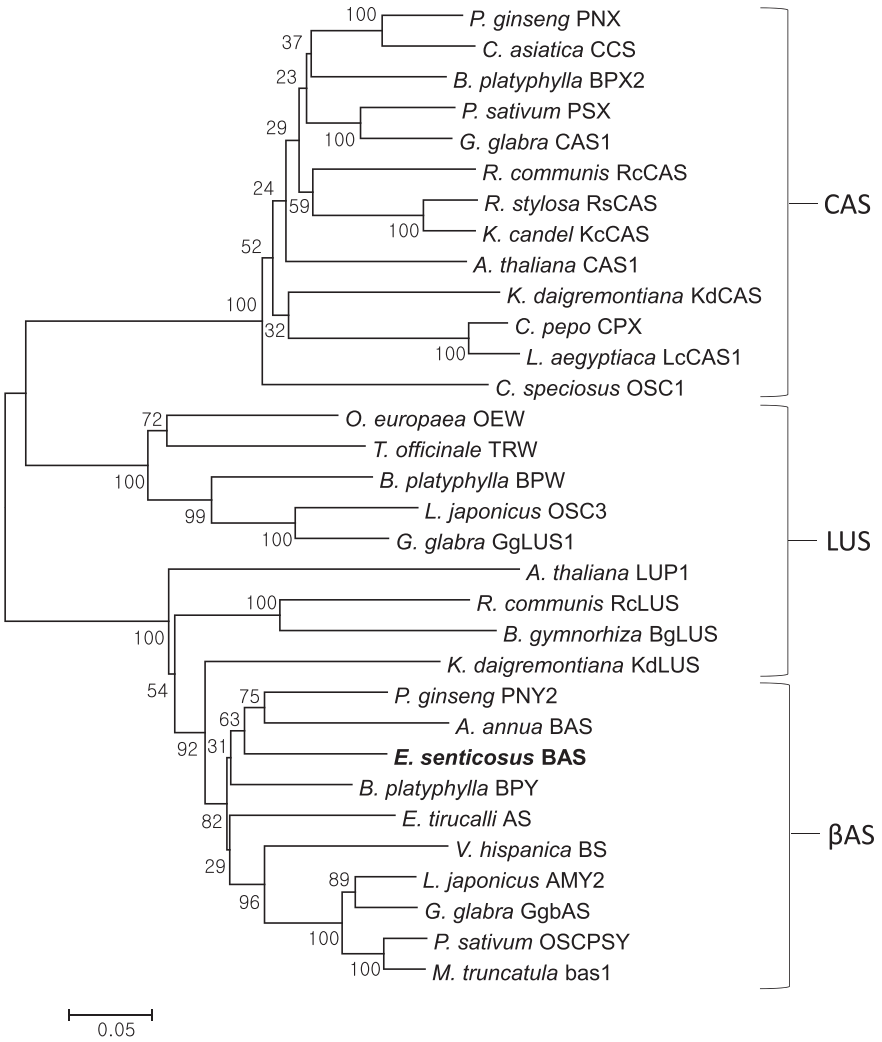

Fig. 2. Phylogenetic tree of the deduced amino acid sequences of EsBAS and other plant OSCs. Phylogenetic trees of the plant OSC distances between each group generated using the neighbour-joining method with the MEGA 5.2 software programme (Tamura et al., 2011). Bootstrap analysis values with 1000 replicates are shown at the nodal branches (Felsenstein, 1985). The indicated scale represents 0.1 amino acid substitutions per site.

retention time of 36.2 min (Fig. 4a), which precisely matched the β-amyrin (**2**) standard (Fig. 4b). Control yeast expressing the empty vector did not show a β-amyrin (**2**) peak (Fig. 4c). The MS fragmentation pattern at 36.2 min in transgenic yeast showed fragmentation peaks at 218 and 189 m/z (Fig. 4d), consistent with the pattern of the β-amyrin (**2**) standard (Fig. 4e). No other new triterpenoid peak was detected in yeast extract expressing *EsBAS* cDNA.

2.2. β-Amyrin production in transgenic tobacco overexpressing *EsBAS*

Transcription of introduced genes in the 6 transgenic tobacco lines was confirmed using RT-PCR. The introduced genes, *hygromycin phosphotransferase* (*HPT*) and *EsBAS*, were obviously transcribed in the transgenic lines, but not in the control (Fig. 5). The leaf extracts of transgenic and non-transgenic control plants were analysed using GC/MS. TIC indicated a new peak at a retention time of 35.8 min in transgenic plants (Fig. 6a), but not in the non-transgenic controls (Fig. 6b). The retention time of the peak (Fig. 6c) and its MS fragmentation pattern (Fig. 6d) precisely matched those of the β-amyrin (**2**) standard (Fig. 6e).

The β-amyrin (**2**) content in the leaves of transgenic lines ranged from 30 to 60 μg/g dry weight, depending on the lines (Fig. 7a). The leaves of one line (T23) of transgenic tobacco were cultured on medium containing different concentrations of 2,4-D (0, 0.1, 0.5, 1.0, and 2.0 mg/l). After 5 weeks of culture, the extracts from the calluses were analysed using GC/MS. The β-amyrin (**2**) content in the callus was highly enhanced after 2,4-D treatment (particularly at 1.0 mg/l 2,4-D) compared with the control (Fig. 7b). β-Amyrin (**2**) accumulation was higher than 20-fold in calluses grown on medium containing 1.0 mg/l 2,4-D compared with that of the control (Fig. 7c). However, the induction and growth of the callus was highest on medium with 0.1 mg/l 2,4-D and gradually declined with increasing 2,4-D concentration (data not shown).

2.3. Characterisation of β-amyrin 28-oxidase (*CYP716A244*) in *E. senticosus*

CYP is a superfamily of monooxygenases, a large and diverse group of enzymes that catalyse the oxidation of organic substances. In transcriptome analysis of the leaf cDNA library of *E. senticosus*, a cDNA sequence (*CYP716A244*) was isolated with high similarity to

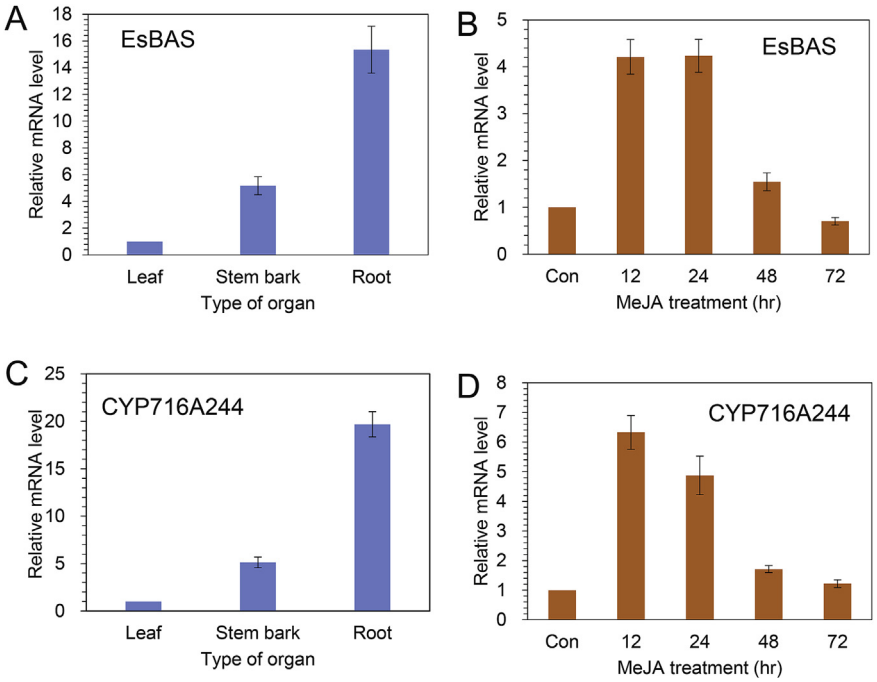

Fig. 3. qPCR analysis of the *EsBAS* and *CYP716A244* genes in different organs (leaf, stem barks, and roots) and in MeJA-treated leaves of *E. senticosus*. **a** The relative fold-expression of the genes in MeJA-treated leaves and untreated controls. The leaves showed the lowest levels of *EsBAS* and *CYP716A244* mRNA transcription and were thus set to 1.0 for relative quantitation. Similarly, the transcript levels of the genes in the control (without MeJA treatment) were set to 1.0. The qPCR results are presented as the means ± standard error (n = 3).

the CYP716A family genes. The full-length cDNA clone of *CYP716A244* is 1721 bp, with an open reading frame (ORF) of 1446 bp, encoding a 481 amino acid protein with a predicted molecular mass of 54.01 kDa. The deduced amino acid sequences of the *CYP716A244* gene showed 89% and 73% identity to the β-amyrin 28-oxidases of *P. ginseng CYP716A52v2* and *M. truncatula CYP716A12*, respectively. Phylogenetic analysis of the deduced amino acid sequence of CYP716A244 and the CYP enzymes of other plant species indicated that CYP716A244 was grouped with the CYP716A subfamily genes (Fig. 8).

qPCR analysis revealed a similar pattern of organ-specific transcription activities for the *CYP716A244* gene compared with that of the *EsBAS* gene (Fig. 3c and d). The quantitative order of *CYP716A244* mRNA accumulation in different plant organs was roots > stem barks > leaves (Fig. 3c). MeJA treatment highly enhanced *CYP716A244* transcription at 12 h after MeJA treatment, and this enhancement declined as the culture time proceeded (Fig. 3d).

To functionally examine the oxidising activity of CYP716A244 of β-amyrin (**2**), both *EsBAS* and *CYP716A244* were co-expressed in yeast expressing *Arabidopsis thaliana* NADPH-CYP reductase (Urban et al., 1997) under control of a galactose-inducible promoter (GAL1). The TIC from liquid chromatograph mass spectrometer-ion trap/time-of-flight (LCMS-IT-TOF) analysis showed that co-expression of *EsBAS* and *CYP716A244* in yeast clearly resulted in three new peaks (Fig. 9a) at retention times of 23.8, 26.5, and 33.9 min, consistent with those of the oleanolic acid (**4**), erythrodiol (**3**) and β-amyrin (**2**) standards, respectively (Fig. 9b). These three signals were not detected in control yeast harbouring the empty vector (Fig. 9c). The MS fragmentation patterns (Fig. 9d) of the signals at these retention times were also identical to those (Fig. 9e) of the respective standards. These results indicated that CYP716A244 is a β-amyrin 28-oxidase that converts β-amyrin (**2**) into oleanolic acid (**4**).

2.4. Co-expression of EsBAS and CYP716A244 in transgenic tobacco

Three transgenic tobacco lines were generated by introducing both the *EsBAS* and *CYP716A244* genes and were confirmed by RT-PCR. The *hygromycin phosphotransferase* (*HPT*), *EsBAS*, and *CYP716A244* genes were expressed in the transgenic lines, but not in the control (Fig. 10a). Leaf extracts of transgenic and non-transgenic plants were analysed using LCMS-IT-TOF. The content of oleanolic acid (**4**), erythrodiol (**3**), and β-amyrin (**2**) ranged from 5.4 to 19.1 μg/g DW, with differences among the transgenic lines (Fig. 10b). The TIC indicated that three new peaks at retention times of 23.8, 26.5, and 33.9 min were also detected in the roots of transgenic tobacco (Fig. 11a), but not in roots of non-transgenic tobacco (Fig. 11b). The three new peaks detected using selected ion mode (SIM) (Fig. 11c) precisely matched those of the authentic oleanolic acid (**4**), erythrodiol (**3**), and β-amyrin (**2**) standards (Fig. 11d). The MS fragmentation pattern (Fig. 11e) of the three new peaks in transgenic tobacco also established the same MS fragmentation (Fig. 8d–f) pattern as that of the authentic standards.

3. Discussion

3.1. Characterisation of the EsBAS gene in E. senticosus

OSCs are encoded by multigene families in plants and catalyse the cyclisation of 2,3-oxidosqualene to generate either sterol or triterpene skeletons. Oleanane-type triterpenes are the major metabolites in *E. senticosus*. The enzyme 2,3-oxidosqualene cyclase (Kushiro et al., 1998) produces β-amyrin (**2**), the basic triterpene backbone of oleanane-type saponin. Thus, β-amyrin (**2**) in *E. senticosus* may play a key role in saponin biosynthesis. In a previous report, many OSC transcripts were identified by 454 pyrosequencing of *E. senticosus* (Hwang et al., 2015). The ectopic expression of the *EsBAS* gene in yeast resulted in β-amyrin (**2**)

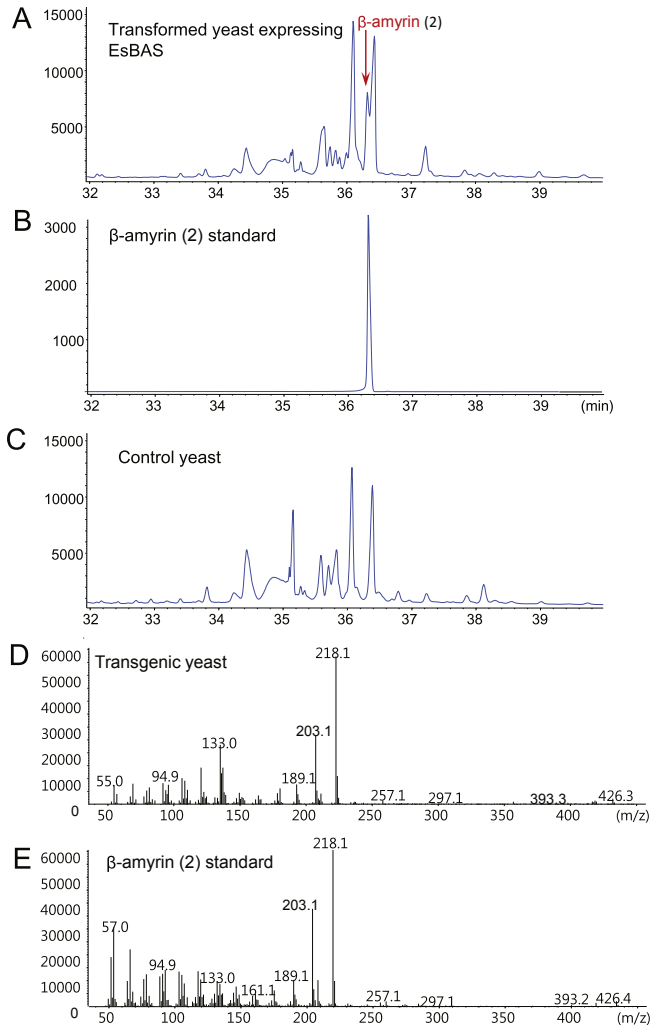

Fig. 4. Total ion chromatogram of the GC/MS analysis of the EsBAS product in yeast. **a** GC chromatography of transgenic yeast cell extract expressing *EsBAS*. **b** GC chromatography of the β-amyrin (**2**) standard. **c** GC chromatography of yeast cell extract with the empty vector as the control. **d** MS spectrum (m/z 426) for a peak detected in transgenic yeast with *EsBAS*. **e** MS spectrum for a peak detected in the β-amyrin (**2**) standard.

production. Several OSC genes encode amyrin synthases that produce mixed triterpenes containing both α- and β-amyrin (**2**) with several minor triterpenes (Thimmappa et al., 2014). In yeasts expressing the *EsBAS* of *E. senticosus*, no mixed triterpenes, except β-amyrin (**2**), were produced.

Most of the functional characterisation of OSC genes has been achieved by heterologous expression of cDNAs in yeast

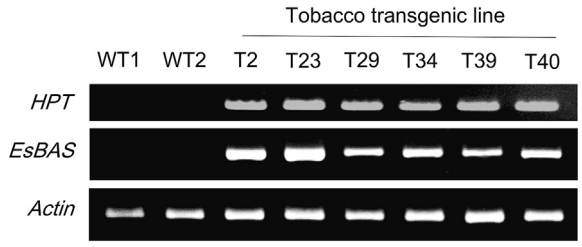

Fig. 5. RT-PCR analysis of the *HPT*, *EsBAS* and *Actin* genes in putative transgenic tobacco plants using *HPT*, *EsBAS* and *Actin* primers; HPT (hygromycin phosphotransferase) was used as a selection marker, and NT actin (*N. tabacum* actin) was used as an internal control.

(Thimmappa et al., 2014). The overexpression of β-amyrin genes in plants is not as common as yeast expression. One article reported that overexpression of the *Panax japonicus* β-amyrin synthase gene in transgenic rice resulted in the production of oleanolic acid (**4**), an oxidation product of β-amyrin (**2**), produced via the introduced β-amyrin synthase (Huang et al., 2015). Tobacco is a model plant for in planta functional analysis of genes of interest and plant metabolic engineering. There is no information on β-amyrin (**2**) production in tobacco. There are generally two strategies for overexpression of the β-amyrin gene in plants. The first strategy is to enhance the accumulation of β-amyrin (**2**) and/or the final saponin products when plants produce β-amyrin (**2**) and β-amyrin-derived saponins (Confalonieri et al., 2009; Pecchia et al., 2011). Another strategy is to newly express β-amyrin (**2**) because the plant does not endogenously express it.

Transgenic tobacco overexpressing *EsBAS* was constructed via *Agrobacterium*-mediated transformation. No β-amyrin (**2**) signal was detected by GC/MS analysis of the non-transgenic tobacco, but a clear β-amyrin (**2**) product was detected in transgenic tobacco overexpressing the *EsBAS* gene. Leaf-derived calluses induced by auxin (2,4-D) treatment contained higher amounts of β-amyrin (**2**) than the leaves of intact plants. These results suggest that β-amyrin (**2**) can be produced in transgenic tobacco overexpressing the *EsBAS* gene and the amount of β-amyrin (**2**) production was more effective via callus culture compared with intact tobacco plants.

3.2. Characterisation of the *E. senticosus CYP716A244* gene

Plant P450s are a large and diverse group of enzymes that play key roles in the production of triterpene aglycone from triterpene skeletons. Until recently, little was known about the types of P450 enzymes involved in triterpene modifications. The basic aglycone of oleanane-type saponins (eleutherosides I, K, L, and M (**5**) and ciwujianosides A1, A3, C3, C4, and D1) in *E. senticosus* is oleanolic acid (**4**) after β-amyrin (**2**) oxidation. β-amyrin 28-oxidase catalyses the oxidation of β-amyrin (**2**) to produce oleanolic acid (**4**). β-amyrin 28-oxidase genes belong to the CYP716A subfamily (Carelli et al., 2011; Fukushima et al., 2011; Han et al., 2013). CYP716A subfamily genes are also involved in the biosynthesis of dammarane-type aglycones in ginseng plants (Han et al., 2011, 2012). In a transcriptomics analysis of the leaf cDNA library of *E. senticosus*, a cDNA sequence (*CYP716A244*) was isolated. The deduced amino acid sequences of the *CYP716A244* gene showed 89% and 73% identity to the β-amyrin 28-oxidase of *P. ginseng CYP716A52v2* and *M. truncatula CYP716A12*, respectively. To functionally characterise *CYP716A244*, this gene was co-expressed with β-amyrin synthase (*EsBAS*) in both yeast and tobacco. Both transgenic yeast and tobacco co-expressing both *EsBAS* and *CYP716A244* genes produced oleanolic acid (**4**), β-amyrin (**2**) and erythrodiol (**3**). Erythrodiol (**3**) production in both yeast and tobacco is consistent with previously reported data, indicating that β-amyrin (**2**) is oxidised in a sequential three-step oxidation reaction at the C-28 position to yield oleanolic acid (**4**) through erythrodiol (**3**) (Fukushima et al., 2011). Both CYP716A12 and CYP716A15 are multifunctional oxidase enzymes with β-amyrin 28-hydroxylase, α-amyrin 28-hydroxylase, and lupeol 28-hydroxylase activities (Carelli et al., 2011; Fukushima et al., 2011). The multifunctional activity of *CYP716A244* remains elusive.

Recent studies have demonstrated that free oleanolic acid (**4**) and ursolic acid have anti-inflammatory, antioxidant, and anti-tumour activities (Banno et al., 2004; Liu, 1995; Sultana and Ata, 2008; Pollier and Goossens, 2012). β-Amyrin (**2**) has also been shown to exhibit anti-microbial, anti-inflammatory and

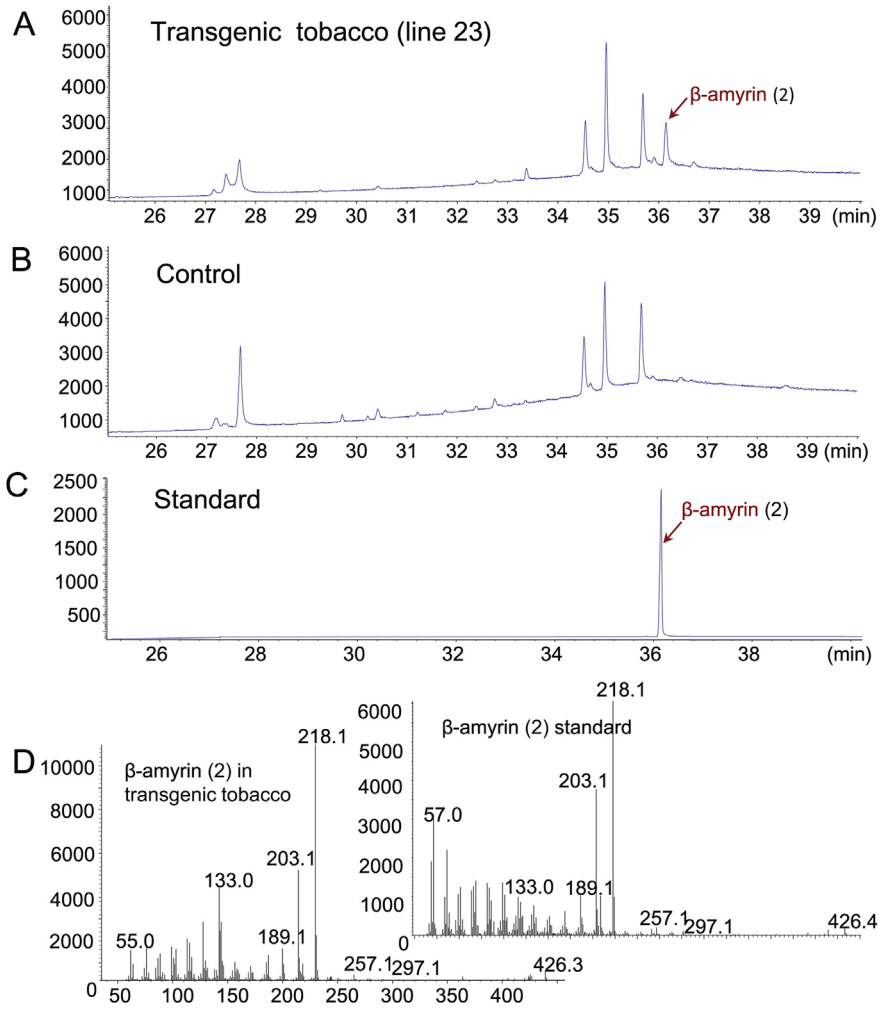

Fig. 6. GC/MS chromatogram of the extracts from the leaves of transgenic tobacco expressing the *EsBAS* gene. **a** GC chromatography of the leaf extracts from the transgenic tobacco plant (line T23). **b** GC chromatography of non-transgenic tobacco leaf extracts as a control. **c** GC chromatography of the β-amyrin (**2**) standard. **d** MS spectrum for the peaks detected in transgenic tobacco expressing the *EsBAS* gene. The insert represents the MS spectrum for a peak detected in the β-amyrin (**2**) standard.

other biological activities. The intermediate ginsenoside aglycones (sapogenin) have stronger biological effects than the ginsenosides (Jia et al., 2004; Popovich and Kitts, 2004). The antitumour activities of deglycosylated saponin (protopanaxadiol) are higher than those of any of the ginsenosides (Bae et al., 2004; Jia et al., 2004; Popovich and Kitts, 2004; Li et al., 2006). In the present study, both β-amyrin (**2**) and oleanolic acid (**4**) were successfully produced in transgenic yeast and tobacco. This result suggests a promising strategy to them using genetic engineering.

4. Conclusion

It was demonstrated that *EsBAS* and *CYP716A244*, isolated from *E. senticosus*, were β-amyrin synthase and β-amyrin 28-oxidase, respectively. β-Amyrin (**2**) and oleanolic acid (**4**) production was achieved by co-expression of both *EsBAS* and *CYP716A244* in transgenic tobacco. Engineered yeast and tobacco expressing *EsBAS* resulted in β-amyrin (**2**) production. Engineered yeast and tobacco co-expressing both *EsBAS* and *CYP716A244* resulted in oleanolic acid (**4**) production. This result is the first report of β-amyrin (**2**) and oleanolic acid (**4**) production in transgenic tobacco.

5. Experimental

5.1. qPCR analysis

RNA was isolated from leaf, stem bark, and root tissues of field-grown *E. senticosus* plants. To analyse the response of *EsBAS* and *CYP716A244* genes to MeJA, young leaves were placed onto 1/3 strength MS medium containing 10 μM MeJA for 72 h. All tissues were immediately frozen in N_2 and stored at −80 °C until further use. Extraction of mRNA, cDNA preparation from mRNA, and qPCR analysis were conducted according to Hwang et al. (2015). The *E. senticosus β-actin* gene was used for normalisation. The following primers were used for amplification: 5′- GTA GCC CGG GAG AGC TAG A -3′ and 5′- GTA GTG GCG GCC TCA AAA GT -3′ for *EsBAS*; 5′- ATG TAC CAC GTT GTG AAG AGG T-3′ and 5′- TTA AAG CAC CAC CCG AAG -3′ for *CYP716A244*; and 5′- CTC GCA TCT CTC AGC ACC TT -3′ and 5′- CCA CAG CCA ACT GAG TTC ACA -3′ for the *E. senticosus* β-actin gene.

5.2. Isolation and expression of EsBAS and CYP716A244 in yeast

Full-length cDNAs of *EsBAS* (GenBank accession number, KX378998) and *CYP716A244* (GenBank accession number,

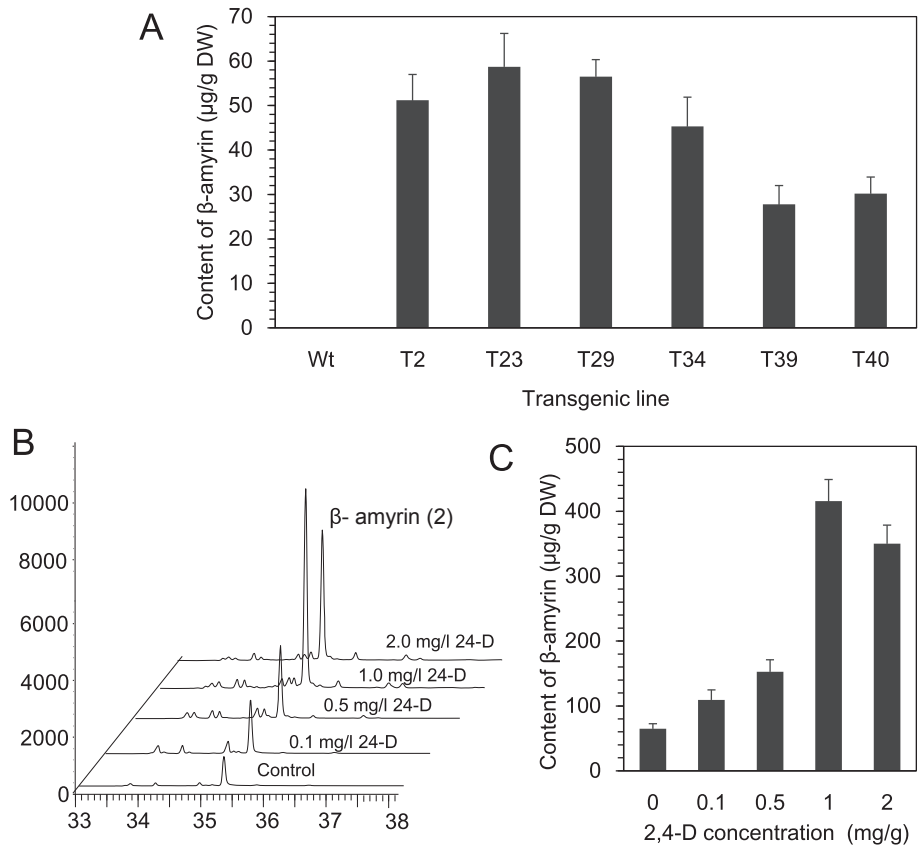

Fig. 7. Production of β-amyrin (**2**) in transgenic tobacco and *in vitro* cultured calluses. **a** Content of β-amyrin (**2**) in the transgenic lines. The data are shown as the means ± standard error obtained from three independent plants. **b** GC chromatograms extracted from calluses cultured on medium containing different concentrations of 2,4-D. **c** β-amyrin (**2**) contents in the leaf-derived calluses of transgenic tobacco (line 23) overexpressing *EsBAS* induced on medium containing different concentrations of 2,4-D. The data are shown as the means ± standard error obtained from three independent plants.

KX354739) were obtained from the ESTs in the leaf transcriptomics library of *E. senticosus*. The ORF of *EsBAS* cDNA was amplified using PCR (30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2.5 min) with Pfu DNA polymerase (TaKaRa Bio, Tokyo, Japan) and subsequently cloned into pYES2.1 using the TOPO TA Expression Kit (Invitrogen, Groningen, The Netherlands). Two primers, 5′- ATG TGG AAG TTG AAG ATA GCG GAA GG -3′ and 5′- CTA ATT AGG CGC CTG GGA CGG TAA TG -3′, were used to isolate the cDNA. The PCR product was ligated downstream of the GAL1 promoter in the sense orientation into pYES2.1/V5-His-TOPO, and the nucleotide sequence of the inserted DNA was confirmed by sequencing. The recombinant vector was used to transform TOP 10F' *Escherichia coli* (Invitrogen) cells. The erg7-deficient yeast (MATa erg7 ura3^{-1} trp1^{-1}) (Karst and Lacroute, 1977) was transformed with pYES2.1/V5-His-TOPO carrying *EsBAS* using the heat shock method for all transformations.

For the co-expression of *EsBAS* and *CYP716A244* in yeast, the ORF for *EsBAS* was amplified by PCR using a primer set with restriction enzyme sites at the 5′ end (NotI- -5′- ATG TGG AAG TTG AAG ATA GCC GAA GG -3′ and SacI-5′- CTA ATT AGG CGC CTG GGA CGG TAA TG −3′), cloned into the pGEM-T Easy vector (Promega) and sequenced. The ORF fragments were excised after digesting with NotI and SacI (TaKaRa) and inserted into the pESC-URA vector harbouring the respective restriction enzyme sites (Agilent Stratagene, USA). Similarly, the coding region of the *CYP716A244* gene was subcloned into the pGEM-T Easy vector as an XhoI-KpnI fragment using the primer pairs XhoI- 5′-CTCGAG ATG CAA CTC TTC TAT GTC CCT CTC-3′ and KpnI-5′-GGTACC TTA GGC TTT GTG AGG GAA CAG GCG-3′. The plasmid DNA was digested with XhoI and KpnI and ligated into the respective sites of the pESC-URA vector harbouring the *EsBAS* gene. The resulting plasmid (pESC*EsBAS*-*CYP716A244*-URA) was transformed into *Saccharomyces cerevisiae*

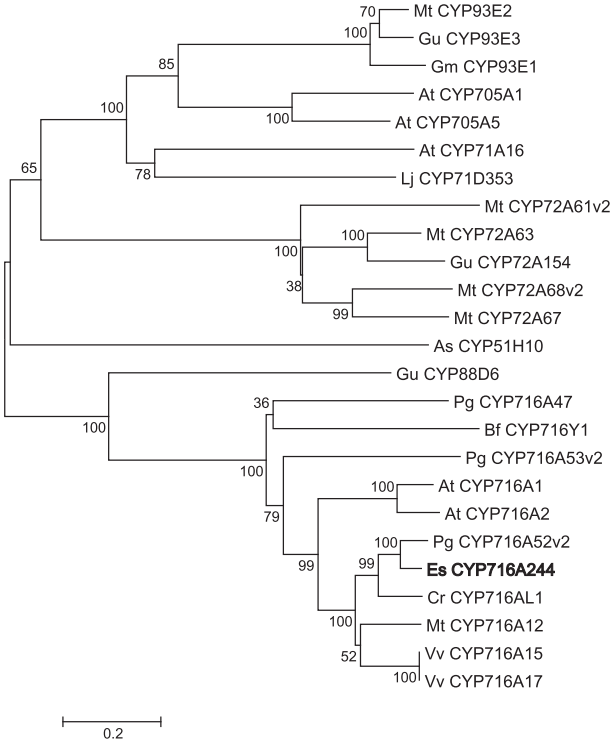

Fig. 8. Phylogenetic tree of the deduced amino acid sequences of CYP716A244 and other plant CYPs. Phylogenetic trees showing the plant OSC distances between each group and clone were generated using the neighbour-joining method with the MEGA 5.2 software programme (Tamura et al., 2011). Bootstrap analysis values with 1000 replicates are shown at the nodal branches. The indicated scale represents 0.1 amino acid substitutions per site.

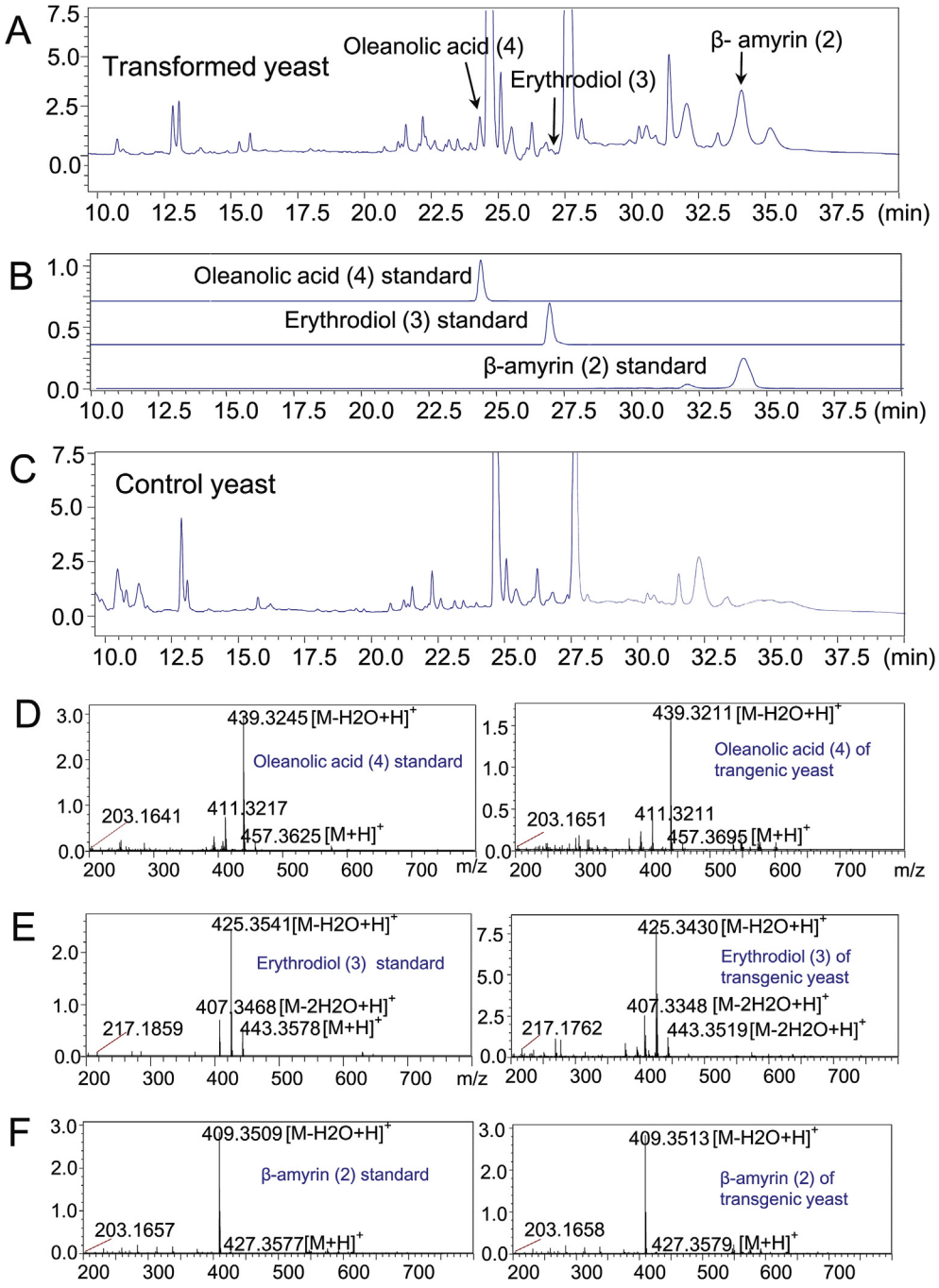

Fig. 9. LCMS-IT-TOP analysis of oleanolic acid (**4**) production in WAT21 yeast expressing both *EsBAS* and *CYP716A244*. **a** TIC of the yeast extracts overexpressing both *EsBAS* and *CYP716A244*; the three arrows indicate oleanolic acid (**4**), erythrodiol (**3**), and β-amyrin (**2**) at retention times of 23.8, 26.5, and 33.9 min, respectively. **b** Total ion chromatogram of oleanolic acid (**4**), erythrodiol (**3**), and β-amyrin (**2**) standards. **c** TIC of the yeast extracts, with an empty vector as a control. **d** MS spectrum of oleanolic acid (**4**), erythrodiol (**3**), and β-amyrin (**2**) peaks from transformed yeasts and those of authentic standards.

strain WAT21, carrying *A. thaliana* NADPH-CYP reductase (Urban et al., 1997), using the same method as described above. The yeast culture conditions, galactose induction and triterpene monoalcohol fraction preparation have previously been described (Han et al., 2013).

5.3. Construction of transgenic tobacco overexpressing EsBAS and co-overexpressing EsBAS and CYP716A244 genes

To construct the binary vector for tobacco transformation, the ORF region of *EsBAS* was cloned using the GATEWAY vector pCR8/GW/TOPO (Invitrogen, USA) and transferred into the binary destination vector, pH2WG7, using LR Clonase Enzyme Mix 2 (Invitrogen, USA), inserting this region downstream of the double 35S CaMV promoter. To construct a vector harbouring both the *EsBAS* and *CYP716A244* genes, the ORFs of both *EsBAS* and *CYP716A244* were amplified and cloned using the GATEWAY vector pCR8/GW/TOPO (Invitrogen, Carlsbad, CA) and subsequently transferred into the destination vector pPZIP-Bar under the control of the double 35S CaMV promoter.

The overexpression constructs harbouring *EsBAS* or both *EsBAS* and *CYP716A244* for tobacco transformation were introduced into *Agrobacterium tumefaciens* GV3101 strain competent cells using a heat shock method. The construction of transgenic tobacco by *A. tumefaciens*-mediated transformation was performed according to Han et al. (2014) using a different selection medium containing BAR.

5.4. RT-PCR analysis

For RT-PCR analysis, total RNA was isolated from tobacco plants using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions and reverse-transcribed using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). First-strand DNA was used as the template for RT-PCR analysis, which was performed as follows: 96 °C for 5 min; 30 cycles at 96 °C for 30 s, 55 °C for 30 s, and 72 °C for 2.5 min; and a final extension at 72 °C for 10 min. The primers used for the *EsBAS* gene were 5′- ATG TGG AAG TTG AAG ATA GCC GAA GG -3′ and 5′- CTA ATT AGG CGC CTG GGA CGG TAA TG -3′, the primers used for the *CYP716A244* gene were 5′ CTTCCTCAAACCGGAAGCCT -3′ and 5′- AGGCTTTGT-GAGGGAACAGC -3′, the primers used for the *HPT* gene were 5′- GCG TGA CCT ATT GCA TCT CC -3′ and 5′-TTC TAC ACA GCC ATC GGT CC-3′, and the primers used for *β-actin* were 5′- ACA GGT ATT GTG TTG GAT TC -3′ and 5′- TGT TGG AAG GTG CTG AGA G -3′. *β-actin* was used as an internal control to assess the RNA integrity and accuracy of loading. Electrophoresis of the products was performed using 1% agarose/0.5 X TBA buffer. The RT-PCR analyses were repeated twice, and representative data are presented in the figures.

5.5. Callus culture of transgenic tobacco cells

The leaves of transgenic tobacco plants were cut into segments and transferred onto Murashige and Shoog (1962) medium containing various concentrations of 2,4-D (0. 0.1, 0.5, 1.0, and 2.0 mg/l), 3% sucrose, 20 mg/l of hygromycin, and 0.27% Gelrite. All media were adjusted to pH 5.8 prior to autoclaving at 120 °C for 15 min. Approximately 15 segments were cultured on a Petri dish for 5 weeks. The culture room was maintained at 22 ± 1 °C under 24-μmol m^{-2} s^{-2} white fluorescence illumination.

5.6. GC/MS analysis of yeast extract and transgenic tobacco overexpressing EsBAS

The hexane extract of yeast harbouring *EsBAS* was evaporated and dissolved in MeOH (1 ml). A 10-μl aliquot of the solution was analysed using GC (Agilent 7890A) linked to an inert MSD system (Agilent 5975C) with a Triple-Axis detector equipped with a HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 mm). Additional details for operating the GC/MS analysis are described in the methods of Han et al. (2013). The standard β-amyrin (**2**) for GC/MS analysis was obtained from Sigma-Aldrich Co.

For GC/MS analysis of transgenic tobacco, milled powder from each of the freeze-dried leaf samples (200 mg) was soaked in hexane. The supernatant collected after centrifugation was dried. After evaporation, the residue was dissolved in MeOH and subsequently filtered using a SepPak C-18 Cartridge (Waters, Milford, MA, USA). All steps of GC analysis were the same as described above.

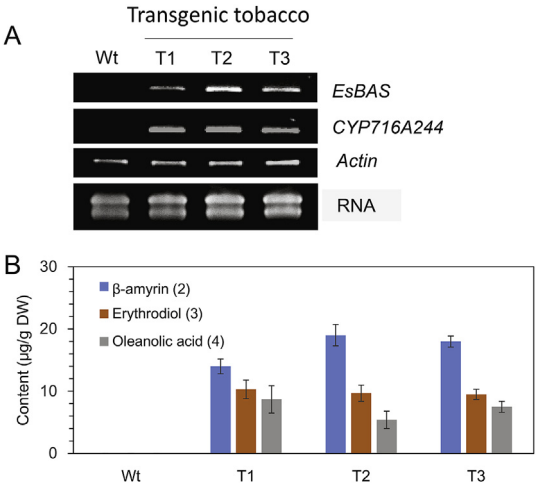

Fig. 10. Detection of the transcription of the introduced genes and metabolite products in transgenic tobacco leaves overexpressing *EsBAS* and *CYP716A244*. **a** Confirmation of the transcription of the introduced genes (*EsBAS*, and *CYP716A244*) using RT-PCR. Actin was used as an internal control. **b** The content of oleanolic acid (**4**), erythrodiol (**3**), and β-amyrin (**2**) in different transgenic lines.

5.7. LCMS-IT-TOF analysis of the transformed yeast and tobacco overexpressing both EsBAS and CYP716A244

Transformed yeast cells (1 g) harbouring *EsBAS* and *CYP716A244* or the empty vector were extracted after sonication with KOH (20%) in EtOH:H_2O (15 ml, 1:1, v/v) for 10 min. For the extraction of transgenic tobacco overexpressing *EsBAS* and *CYP716A244,* milled powder (200 mg) from freeze-dried leaf samples was soaked in MeOH. The supernatant was collected after centrifugation. After evaporation, the residue was dissolved in MeOH, and subsequently filtered using a SepPak C-18 Cartridge (Waters, Milford, MA, USA).

LC analyses were conducted on a Shimadzu (Kyoto, Japan) HPLC system comprising an LC-20AD binary pump, DGU-20A degasser, SIL-20A autosampler and a CTO-20AC column oven, with an SPD-M20A PDA detector. The mobile phase (delivered at 0.5 ml/min) was composed of solvent A (H_2O) and solvent B (CH_3CN). A binary gradient elution for β-amyrin (**2**), oleanolic acid (**4**), and erythrodiol (**3**) was performed: initial 25% B for 5.0 min, 25–55% B from 5 min to 10 min and 55–70% B from 10 min to 15 min, and 70–90% B from 15 min to 20 min, and 90–100% B from 20 min to 30 min, and 100-75% B from 30 min to 35 min, returned to initial 25% B and maintained until 40 min for column balance. Chromatographic separation was achieved on a YMC-Pack Pro C18 RS column (150 × 2.0 mm.D, S-5 μm, 8 nm, YMC Co., LTD. JAPAN) at 40 °C.

A LCMS–IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan) was equipped with an APCI source in positive and negative ion modes. The following optimised analytical conditions were used: detector voltage, 1.60 kV; nebulising gas (N2) flow, 1.5 L/min; dry gas (N2) flow, 50 kPa; pressure of TOF region, 1.5×10^{-4} Pa; ion trap pressure, 1.7×10^{-2} Pa; ion accumulated time, 30 ms; and precursor ion selected width, 3.0 amu. For qualitative analysis, the scan ranges were set at m/z 100–1000 for MS1 and 100–500 for MS2; ultra-high purity Ar was used as the cooling gas and the collision gas for CID experiments, and the collision energy was set at 50% for MS2. Authentic β-amyrin (**2**), oleanolic acid (**4**), and erythrodiol (**3**) were directly analysed using the same conditions. Their amounts were quantified by calculating the ratio of the peak area of the respective compounds to those of the standards.

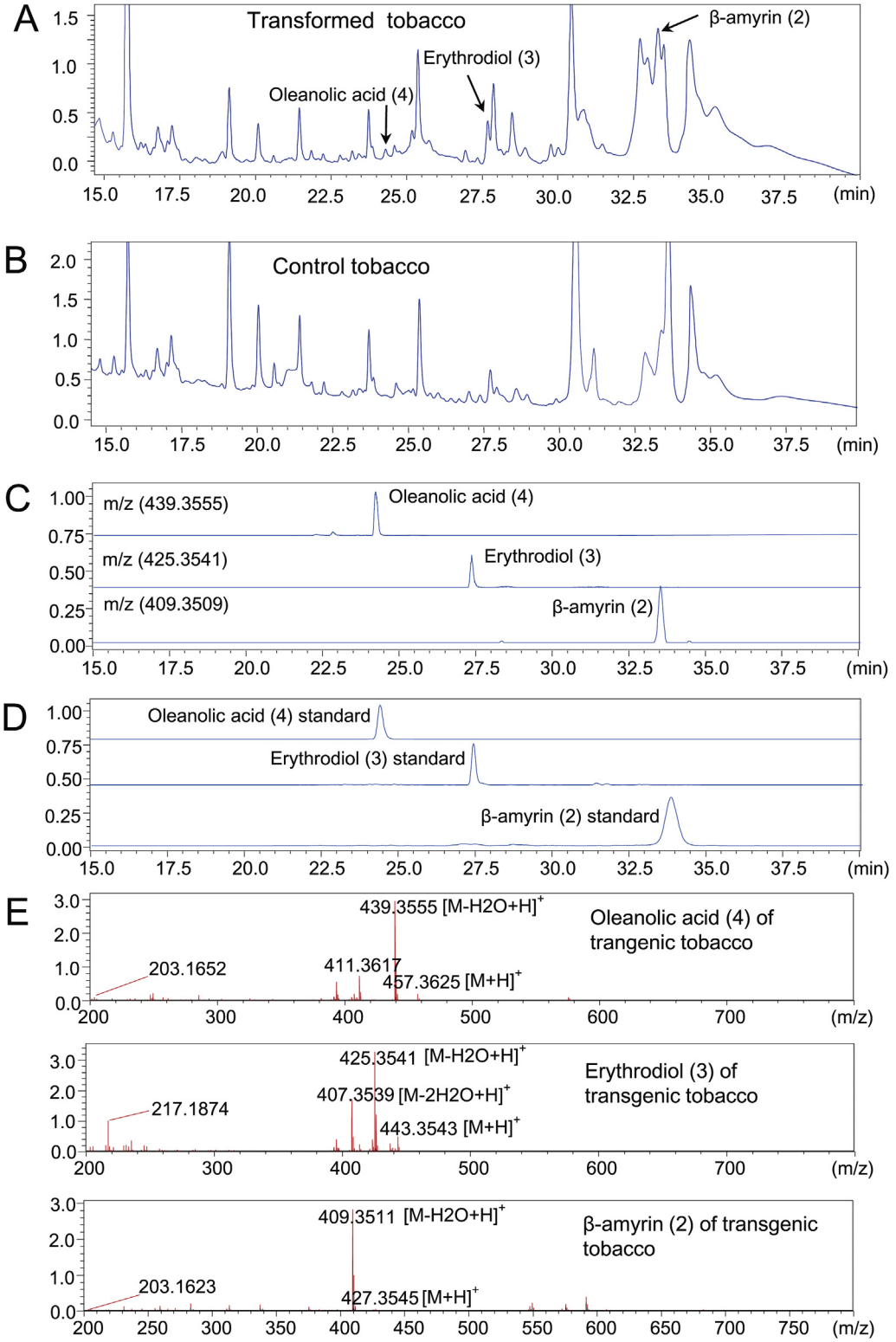

Fig. 11. LCMS-IT-TOP analysis of oleanolic acid production in transgenic tobacco co-overexpressing both *EsBAS* and *CYP716A244*. **a** TIC of transgenic tobacco (line 23); the three arrows indicate oleanolic acid (**4**), erythrodiol (**3**), and β-amyrin (**2**) at retention times of 23.8, 26.5, and 33.9 min, respectively. **b** TIC of the non-transgenic control. **c** Distinct detection of oleanolic acid (**4**), erythrodiol (**3**), and β-amyrin (**2**) at retention times using single ion mode chromatography of the extracts from transgenic tobacco co-overexpressing both *EsBAS* and *CYP716A244*. (D) TIC of oleanolic acid (**4**), erythrodiol (**3**), and β-amyrin (**2**) standards. **d** MS spectrum of oleanolic acid (**4**), erythrodiol (**3**), and β-amyrin (**2**) peaks from transformed tobacco.

Author contributions

YEC designed the study and drafted the manuscript. JHC, JYH, and HSH prepared the figures and performed RT-PCR.

Conflict of interest

The authors declare that they have no conflicts of interest.

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References

Abe, I., Rohmer, M.D., Prestwich, G., 1993. Enzymatic cyclization of squalene and oxidosqualene to sterols and triterpenes. Chem. Rev. 93, 2189–2206.
Bae, E.A., Han, M.J., Kim, E.J., Kim, D.H., 2004. Transformation of ginseng saponins to ginsenoside Rh2 by acids and human intestinal bacteria and biological activities of their transformants. Arch. Pharm. Res. 27, 61–67.
Banno, N., Akihisa, T., Tokuda, H., Yasukawa, K., Higashihara, H., Ukiya, M., Watanabe, K., Kimura, Y., Hasegawa, J., Nishino, H., 2004. Triterpene acids from the leaves of *Perilla frutescens* and their antiinflammatory and antitumor-promoting effects. Biosci. Biotechnol. Biochem. 68, 85–90.
Carelli, M., Biazzi, E., Panara, F., Tava, A., Scaramelli, L., Porceddu, A., Graham, N., Odoardi, M., Piano, E., Arcioni, S., May, S., Scotti, C., Calderini, O., 2011. *Medicago truncatula* CYP716A12 is a multifunctional oxidase involved in the biosynthesis of hemolytic saponins. Plant Cell 23, 3070–3081.
Confalonieri, M., Cammareri, M., Biazzi, E., Pecchia, P., Fevereiro, S., Balestrazzi, A., Tava, A., Conicella, C., 2009. Enhanced triterpene saponin biosynthesis and root nodulation in transgenic barrel medic (*Medicago truncatula* Gaertn.) expressing a novel β-amyrin synthase (*AsOXA1*) gene. Plant Biotech. J. 2, 172–182.
Davydov, M., Krikorian, A.D., 2000. *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. (Araliaceae) as an adaptogen: a closer look. J. Ethnopharmacol. 72, 345–393.
Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.
Fukushima, E.O., Seki, H., Ohyama, K., Ono, E., Umemoto, N., Mizutani, M., Saito, K., Muranaka, T., 2011. CYP716A subfamily members are multifunctional oxidases in triterpenoid biosynthesis. Plant Cell Physiol. 52, 2050–2061.
Han, J.Y., Kim, H.J., Kwon, Y.S., Choi, Y.E., 2011. The Cyt P450 enzyme CYP716A47 catalyzes the formation of protopanaxadiol from dammarenediol-II during ginsenoside biosynthesis in *Panax ginseng*. Plant Cell Physiol. 52, 2062–2073.
Han, J.Y., Hwang, H.S., Choi, S.W., Kim, H.J., Choi, Y.E., 2012. Cytochrome P450 *CYP716A53v2* catalyzes the formation of protopanaxatriol from protopanaxadiol during ginsenoside biosynthesis in *Panax ginseng*. Plant Cell Physiol. 53, 1535–1545.
Han, J.Y., Kim, M.J., Ban, Y.W., Hwang, H.S., Choi, Y.E., 2013. The involvement of β-amyrin 28-oxidase (CYP716A52v2) in oleanane-type ginsenoside biosynthesis in *Panax ginseng*. Plant Cell Physiol. 54, 2034–2046.
Han, J.Y., Wan, H.Y., Choi, Y.E., 2014. Production of dammarenediol-II triterpene in a cell suspension culture of transgenic tobacco. Plant Cell Rep. 33, 225–233.
Huang, Z., Lin, J., Cheng, Z., Xu, M., Guo, M., Huang, X., Yang, Z., Zheng, J., 2015. Production of oleanane-type sapogenin in transgenic rice via expression of β-amyrin synthase gene from *Panax japonicus* C. A. Mey. BMC Biotechnol. 15, 45.
Hwang, H.S., Lee, H.S., Choi, Y.E., 2015. Transcriptomic analysis of Siberian ginseng (*Eleutherococcus senticosus*) to discover genes involved in saponin biosynthesis. BMC Genom. 16, 180.
Jia, W., Yan, H., Bu, X., Liu, G., Zhao, Y., 2004. Aglycone protopanaxadiol, a ginseng saponin, inhibits P-glycoprotein and sensitizes chemotherapy drugs on multi-drug resistant cancer cells. ASCO annual meeting proceedings (post-meeting edition). J. Clin. Oncol. 22, 9663. Suppl.
Karst, F., Lacroute, F., 1977. Ergosterol biosynthesis in *Saccharomyces cerevisiae* mutants deficient in the early steps of the pathway. Mol. Gen. Genet. 154, 269–277.
Kushiro, T., Shibuya, M., Ebizuka, Y., 1998. β-Amyrin synthase: cloning of oxidosqualene cyclase that catalyzes the formation of the most popular triterpene among higher plants. Eur. J. Biochem. 256, 238–244.
Lambert, E., Faizal, A., Geelen, D., 2011. Modulation of triterpene saponin production: in vitro cultures, elicitation, and metabolic engineering. Appl. Biochem. Biotechnol. 164, 220–237.
Li, G., Wang, Z., Sun, Y., Liu, K., Wang, Z., 2006. Ginsenoside 20(S)-protopanaxadiol inhibits the proliferation and invasion of human fibrosarcoma HT1080 Cells. Basic Clin. Pharmacol. Toxicol. 98, 588–592.
Liu, J., 1995. Pharmacology of oleanolic acid and ursolic acid. J. Ethnopharmacol. 49, 57–68.
Murashige, T., Shoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Planta 3, 473–497.
Pecchia, P., Cammareri, M., Malafronte, N., Consiglio, M.F., Gualtieri, M.J., Conicella, C., 2011. Quinic acids from *Aster caucasicus* and from transgenic callus expressing a beta-amyrin synthase. Nat. Prod. Commun. 6, 1665–1669.
Pollier, J., Goossens, A., 2012. Oleanolic acid. Phytochemistry 77, 10–15.
Popovich, D.G., Kitts, D.D., 2004. Ginsenosides 20(S)-protopanaxadiol and Rh2 reduce cell proliferation and increase sub-G1 cells in two cultured intestinal cell lines (Int-407 and Caco-2). Can. J. Physiol. Pharmacol. 82, 183–190.
Sultana, N., Ata, A., 2008. Oleanolic acid and related derivatives as medicinally important compounds. J. Enzyme Inhib. Med. Chem. 23, 739–756.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
Thimmappa, R., Geisler, K., Louveau, T., O'Maille, P., Osbourn, A., 2014. Triterpene biosynthesis in plants. Annu. Rev. Plant Biol. 65, 225–257.
Umeyama, A., Shoji, N., Takei, M., Endo, K., Arihara, S., 1992. Ciwujianosides D1 and C1: powerful inhibitors of histamine release induced by anti-immunoglobulin E from rat peritoneal mast cells. J. Pharm. Sci. 81, 661–662.
Urban, P., Mignotte, C., Kazmaier, M., Delorme, F., Pompon, D., 1997. Cloning, yeast expression, and characterization of the coupling of two distantly related *Arabidopsis thaliana* NADPH-cytochrome P450 reductases with P450 CYP73A5. J. Biol. Chem. 272, 19176–19186.
Xu, R., Fazio, G.C., Mutsuda, S.P., 2004. On the origins of triterpenoid skeletal diversity. Phytochemistry 3, 261–291.