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Production of Multiple triterpenes Including Taraxasterol in Transgenic Tobacco Overexpressing Multifunctional Oxidosqualene Cyclase (TcOSC1) of Taraxacum Coreanum

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Research Article

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Abstract

Taraxasterol and ψ-taraxasterol are pentacyclic triterpenoids, they are commonly found in the family Asteraceae. These two compounds are useful candidates for pharmacologically active triterpenes in dandelion. A multifunctional oxidosqualene cyclase (TcOSC1) of *Taraxacum coreanum* catalyzes the cyclization of 2,3-oxidosqualene into various triterpenes (taraxasterol, ψ-taraxasterol, δ-amyrin, β-amyrin, α-amyrin, and dammarendiol-II). Here, we established the production of taraxasterol, ψ-taraxasterol, δ-amyrin, β-amyrin, and α-amyrin in transgenic tobacco overexpressing *TcOSC1* gene of *T. coreanum*. Transgenic tobacco overexpressing *TcOSC1* gene was induced via *Agrobacterium*-mediated transformation, and four transgenic lines were selected. Introduction and expression of transgenic genes in tobacco was confirmed by genomic PCR, and qRT-PCR, respectively. All the four transgenic lines of tobacco produced obviously the five triterpenes, namely taraxasterol, ψ-taraxasterol, δ-amyrin, β-amyrin, and α-amyrin. Organ-specific triterpene accumulation occurred in transgenic tobacco plants (leaf > stem > root). The amount of taraxasterol was found the highest among the five triterpenes produced in tobacco. The total amount of triterpenes in transgenic line 3 (Tr3) exhibiting the highest amount of triterpenes that was 598 μg g^{-1} dry weight. Production of phytosterols (β-sitosterol, campesterol, and stigmasterol) was reduced in transgenic tobacco compared to those of wild-type control. Conclusively, we successfully established the production of taraxasterol and ψ-taraxasterol triterpenes in transgenic tobacco, which can be applied to the cost-effective production for the utilization and as a source of pharmacologically active materials.

Introduction

Plants contain a highly diverse group of triterpenes, and are categorized according to the number and structure of the triterpene rings (Xu et al. 2004; Connolly 2010). Triterpenes exist in the free state and/or conjugated form, such as triterpene glycosides (saponin) or triterpene esters. Triterpenes in plants possess allelopathic, antimicrobial, antifungal, insecticidal, and antifeedant activities against herbivores (González-Coloma et al. 2011). Moreover, they also have many biological activities in animals and may have important medicinal activities in humans (Battineni et al. 2018).

Triterpenoids are biosynthesized via the mevalonic acid pathway. Production of diverse triterpenes is determined by enzymes of 2,3-oxidosqualene cyclase (OSC) which catalyse the cyclization reaction of 2,3-oxidosqualene to form the diverse types of triterpene skeleton (Figure 1). Now, various members of OSC genes have been functionally characterized in plants (Thimmappa et al. 2014; Ghosh 2016).

Asteraceae (or Compositae) is a very large family of flowering plants. Many species in Asteraceae are economically important plants, such as dandelion, lettuce, sunflower, artichokes, and chrysanthemums. Several triterpenes, such as taraxasterol, ψ-taraxasterol, taraxerol, α- and β-amyrin, and their acetates, have been isolated from dandelion (*Taraxacum officinale*) (Akashi et al. 1994; González-Castejón et al. 2012). Interestingly, taraxasterol and ψ-taraxasterol are commonly occur in various species of Asteraceae family. Taraxasterol has many important pharmacological actions, including antiallergic (Liu et al. 2013),

anti-inflammatory (Zhang et al. 2012; Piao et al. 2015), antioxidant (Xu et al. 2018), and antitumor activities (Takasaki et al. 1999; Ovesná et al. 2004). Recently, several OSC genes were functionally characterized in *Taraxacum coreanum* (Han et al. 2019), Russian dandelion (*Taraxacum koksaghyz*) (Pütter et al. 2019), and Lettuce (Choi et al. 2020). In all the three species, both taraxasterol and ψ-taraxasterol biosynthesis are produced by a multifunctional triterpene synthase. Korean dandelion TcOSC1 catalyzed the production of taraxasterol, Ψ-taraxasterol, α- amyrin, β- amyrin, δ-amyrin, and dammarenediol-II (Han et al. 2019). *T. kok-saghyz* TkOSC1 is able to synthesize taraxasterol, α-amyrin, β-amyrin, and lup-19(21)-en-3-ol (Pütter et al. 2019). Lettuce LsOSC1 can be able to produce five triterpenes (α-amyrin, β-amyrin, ψ-taraxasterol, taraxasterol, and dammarenediol-II) (Choi et al. 2020).

Genetic engineering of plants is useful technology for producing valuable secondary compounds (Verpoorte and Memelink 2002; Wu and Chappell 2008; Kowalczyk et al. 2020). Production of triterpenes from other plant species or microbial hosts via metabolic engineering might be a promising technology for cost-effective production. In this work, we constructed transgenic tobacco overexpressing a multifunctional triterpene synthase (*TcOSC1*) of Korean dandelion and analyzed the production of taraxasterol, ψ-taraxasterol, α-amyrin, β-amyrin, and δ-amyrin in transgenic tobacco.

Materials And Methods

Construction of overexpression vector harboring *TcOSC1* gene

The full-length sequence of *T. coreanum TcOSC1* gene (GenBank accession number, MK351896) was isolated by PCR using primers 5'- ATG TGG AAG CTG AGA ATA GGT GA-3' and 5'- TTA GGT TTC TTG TTT TGG TAA C -3'. The open reading frame of *TcOSC1* was cloned via GATEWAY vector pCR8/GW/TOPO (Invitrogen) and transferred into binary destination vector pB7WG2, placing it under the control of the CaMV 35S promoter (Fig. 2A). Eventually, the overexpression constructs harboring *TcOSC1* was inserted into *Agrobacterium tumefaciens* LBA4404 strain by the heat shock method.

Production of transgenic tobacco overexpressing *TcOSC1* gene by *A. tumefaciens*-mediated transformation

A. tumefaciens harboring the overexpression vectors were cultured for 48 h at 25°C in a yeast exact broth (YEB) medium in a gyratory shaker at 200 rpm. The bacterial suspension (absorbance of approximately 0.6 at 600 nm) was re-suspended in hormone-free MS (Murashige and Skoog 1962) liquid medium after centrifugation for 5 min at 5000 rpm.

Leaf segments from the *in vitro* grown tobacco plants (*Nicotiana tabacum*, cv. Xanthi) were immersed in the bacterial suspension containing 20 μM acetosyringone for 10 min. They were blotted on sterile filter paper and then transferred onto a co-culture medium (MS medium with 2 mg/l BA and 0.5 mg/l IBA).

After 3 days of co-culture incubation in darkness, leaf segments were transferred onto a medium with 500 mg/l cefotaxime and kept in a culture room at 24 ± 2°C with a 16 h photoperiod of 50 μmol m^{-2} s^{-1} light. After 4 weeks of culture, leaf pieces containing early developmental stage of adventitious shoots were transferred onto a selection medium containing 300 mg/l cefotaxime and 20 mg/l bialaphos. Bialaphos-resistant shoots (approximately height: 1–2 cm) were transferred to a new selection medium and maintained by consecutive subculture by 3 week intervals on 1/2 MS medium containing 20 mg/l bialaphos. Transgenic plantlets with roots were transferred to the greenhouse after acclimatization.

Genomic PCR analysis

Genomic DNAs from the leaf tissues of putative transgenic plants and wild-type plants were extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The primers used to amplify the *TcOSC1* gene were 5'-ATG TGG AAG CTG AAG GTT GCT CAA GGA-3', 5'-TTA AAT TTT GAG CTG CTG GTG CTT AGG C-3', and the primers used to amplify the *BAR* gene were 5'-GCG TGA CCT ATT GCA TCT CC-3' and 5'-TTC TAC ACA GCC ATC GGT CC-3'.

qRT-PCR analysis

Total RNAs were isolated from leaves of wild-type and transgenic tobacco using the RNeasy plant mini kit (Qiagen, Hilden, Germany) and reverse-transcribed using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). The primers used for the *TcOSC1* gene were 5'- GAA ACC TAA ACT CCA TTT TAG TGA A -3' and 5'- GCC CGT GAT GAG AGT TTG TA-3'. The primers for tobacco *β-actin* gene were 5'- GCG ACG GTG TCT CAC ATA CA -3' and 5'- ACG TAC ATG GCG GGA ACA TT-3' that was used as the control to check for RNA quality. qPCR data are presented as the mean ± standard error, and the experiments were repeated for three times.

GC-MS analysis of triterpene and phytosterol production in transgenic tobacco

One hundred mg of milled powder from air-dried samples (leaves, stems, roots) was extracted in 100% methanol by sonication for 30 min at a frequency of 20 kHz at 25°C. The supernatant obtained by centrifugation at 15,000 rpm was filtered using a SepPak C-18 cartridge (Waters, Milford, MA, USA). A 10-μl aliquot was analyzed by Agilent 7890A gas chromatography system linked to an Agilent 5975C inert MSD system with a Triple-Axis detector, and equipped with a HP-5MS capillary column (30 m×0.25 mm i.d.,film thickness 0.25 μm). The injection port was 250°C, and the column oven temperature program was 150°C for 5 min, followed by a rise to 300°C at a rate of 5°C min^{-1} and a hold at 300°C for 20 min. The flow rate of He (as carrier gas) was 1.2 ml min^{-1}. The temperature at the interface was 300°C with a split injection (10:1). The ionization chamber was set at 250°C, and electron impact (EI) ionization operated at

70 eV. Identification of all GC chromatogram peaks were made by comparison of their retention times and mass fraction patterns with those of authentic standards. The α-amyrin and β-amyrin standards were obtained from Sigma – Aldrich Inc. (Saint Louis, MO, USA), and δ-amyrin, ψ-taraxasterol, and taraxasterol were obtained from Toronto Research Chemicals Inc. (North York, Canada).

Results

Production of transgenic tobacco overexpressing *TcOSC1* gene

Transgenic tobacco plants overexpressing *TcOSC1* gene (MK351896.1) under the control of the CaMV 35S promoter were constructed (Fig. 2A). Four independent transgenic lines were finally selected. Integration of T-DNA into the tobacco genome was confirmed by PCR of genomic DNA. All the four transgenic lines showed the expected PCR products for the *BAR* and *TcOSC1* genes (Fig. 2B, C). No PCR signal was detected in wild-type tobacco (Fig. 2B,C).

cDNAs extracted from leaves of wild-type and transgenic plants were subjected to qPCR analysis to detect the expression of *TcOSC1* gene. All four transgenic lines showed a clear accumulation of *TcOSC1* mRNA (Fig. 3). In particular, Tr3 line exhibited the highest transcription of *TcOSC1* compared to the other lines (Fig. 3).

Triterpene analysis in the lines of transgenic tobacco overexpressing *TcOSC1*

To analyze the triterpene production driven by overexpression of *TcOSC1* in transgenic tobacco, the third leaves from the top were analyzed by GC/MS. All the four transgenic lines (Tr1, Tr2, Tr3, and Tr4) showed several new triterpene products (taraxasterol, ψ-taraxasterol, α-amyrin, β-amyrin, and δ-amyrin) at the retention times between 35 min to 38 min compared to the wild-type control (Fig. 5B-E). In the wild-type plants, no signal for the five triterpenes was detected except the phytosterols belonging to the triterpene family (Fig. 5A). Identification of triterpenes was confirmed by matching the retention times of triterpene standards (Fig. 5F) with those in transgenic tobacco leaf extracts (Fig. 5B-E). The MS spectra of the five triterpene products revealed that the fragmentation patterns of each triterpenes product in transgenic tobacco were completely matched to the MS spectra of authentic five triterpene standards (Supplementary Fig. 1). In all transgenic tobacco plants, the total amount of triterpenes in the leaves was 401.3 to 598.7 μg g^{-1} DW (Fig. 6A). The content of taraxasterol showed the highest amount among other triterpenes (Fig. 6A). The order of triterpene accumulation was taraxasterol > ψ-taraxasterol > β-amyrin > α-amyrin > δ-amyrin in all four transgenic lines (Fig. 6A).

Triterpene analysis in different parts of transgenic tobacco overexpressing *TcOSC1*

Accumulation of triterpenes in different portion (leaf, stem, and root) of transgenic tobacco (line 3) was analyzed. Triterpene accumulation occurred in an organ-specific manner (leaves > stems > roots) (Fig. 4). Leaves contained the highest amount of triterpenes compared to other roots and stems. In roots, taraxasterol, ψ-taraxasterol, β-amyrin occurred at the detectable amount, but α-amyrin and δ-amyrin existed traceable amount (Fig. 4).

Phytosterol analysis in the lines of transgenic tobacco overexpressing *TcOSC1*

The production of new triterpenes in transgenic tobacco resulted in the negative relationship in the accumulation of phytosterols (Figs. 5A,6B). GC chromatogram revealed that the peak heights of three phytosterols (β-sitosterol, campesterol, and stigmasterol) in leaves of wild-type tobacco (Fig. 5A) were conspicuously higher than those of transgenic lines (Fig. 5B-E). In all four transgenic lines, accumulation of phytosterols (β-sitosterol, campesterol, and stigmasterol) was clearly decreased compared to those of wild-type (Fig. 6B). However, there was no apparent phenotypic difference in the general plant statures among wild-type and transgenic plants, and all transgenic lines of tobacco successfully set seeds.

Discussion

Functional characterization of OSC genes has been mainly achieved by the expression of the gene in *S. cerevisiae*. Some OSCs are identified as multifunctional enzymes with mixed end-products. However, the mechanism of generating mixed-products is unclear, it is probably due to the deprotonation of numerous sites during the cyclization process (Thimmappa et al. 2014). *T. coreanum* TcOSC1 enzyme was characterized as multifunctional triterpene synthase by heterologous expression in yeast (Han et al. 2019). The enzyme can be able to produce 6 triterpenes, taraxasterol and ψ-taraxasterol as major compounds, and α-amyrin, β-amyrin, δ-amyrin, and dammarenediol-II as minor ones (Han et al. 2019). In the present work, we constructed the transgenic tobacco overexpressing *T. coreanum TcOSC1* and observed the production of triterpenes in transgenic tobacco plants. The transgenic tobacco overexpressing *TcOSC1* produced five triterpenes taraxasterol; Ψ-taraxasterol; α-, β-, and δ-amyrin, but not dammarenediol-II. Taraxasterol; Ψ-taraxasterol; α-, β-, and δ-amyrin are pentacyclic triterpene, but dammarenediol-II is tetracyclic triterpene. It is unclear why dammarenediol-II is not detected in transgenic tobacco overexpressing *TcOSC1*. In the yeast overexpressing *TcOSC1*, production of dammarenediol-II is the one of minor production compared to other triterpenes. Undoubtedly, no detection of dammarenediol-II in the transgenic tobacco overexpressing *TcOSC1* might result from below the detection limits in the samples.

In the transgenic yeast expressing *TcOSC1*, taraxasterol was the major compound among other 6 triterpene products (Han et al. 2019). Similarly, transgenic tobacco overexpressing *TcOSC1* showed the production taraxasterol as a major triterpene compound. Thus cyclization pattern of oxidosqualene into triterpenes is similar although expression of *TcOSC1* occurs in the different host organism.

Triterpene production in transgenic tobacco overexpressing *TcOSC1* showed the organ-specific pattern. The accumulation of triterpenes in roots was shallow compared to leaf and stem. In transgenic tobacco overexpressing *P. ginseng* triterpene synthase (*PgDDS*, dammarnediol-II synthase), the order of dammarenediol-II accumulation in organs was roots> stems> leaves> flower buds (Han et al. 2015). In transgenic tobacco overexpressing *P. ginseng PgDDS* (dammarnediol-II synthase) and *CYP716A47* (protopanaxadiol synthase), the proportion of protopanaxadiol to dammarenediol-II varied among the organs, and the accumulation of dammarenediol-II and protopanaxadiol in the transgenic line occurred in an organ-specific manner (roots>leaves>stems>petioles) (Chun et al. 2015). Interestingly, different pattern of organ-specific triterpene accumulation occurred in transgenic tobacco overexpressing *TcOSC1*, although the *TcOSC1* was expressed by constitutive CaMV35S promoter. These results indicate that the organ-specific accumulation of triterpenes is different depended on the produced triterpene-type. The organ-specific accumulation of triterpenes might be either caused by the different flux of triterpene precursors in tobacco plants and/or the different activity of triterpene carriers in tobacco. It is known that translocation or transport of terpenoids is involved in plant ATP-binding cassette transporters (ABC) transporters (Theodoulou 2000; Hwang et al. 2016). Within the plant, ABC transporter family, pleiotropic drug resistance (PDR) transporters play essential functions, such as in hormone transport or defense against biotic and abiotic stresses (Nuruzzaman et al. 2014). NtPDR1 transports the compounds involved in both diterpenes and sesquiterpenes in plants (Pierman et al. 2017). AaPDR3 is involved in sesquiterpene transport (Fu et al. 2017). Triterpene accumulation in different tissues of transgenic tobacco might be affected by the intrinsic activity of ABC transporters or other types of triterpene carriers in tobacco.

Reduced phytosterols in transgenic tobacco producing multiple triterpenes

In this work, new triterpene production in transgenic tobacco plants resulted in decreased accumulation of phytosterols (β-sitosterol, campesterol, and stigmasterol). A similar reduction of phytosterols occurred in transgenic tobacco producing dammarenediol-II (Han et al. 2014). The reduced phytosterol accumulation in transgenic tobacco might be caused by competition for precursors, as both triterpenes and phytosterols in plants are synthesized from the cyclization of their common precursor, 2,3-oxidosqualene (Thimmappa et al. 2014). Thus, cyclization of 2,3-oxidosqualene for the production of multiple pentacyclic triterpenes in transgenic tobacco overexpressing *TcOSC1* can restrain the metabolic flow of phytosterol biosynthesis, which might result in the reduced sterol accumulation observed in transgenic tobacco.

Abbreviations

Bar: Basta resistance gene; GC/MS: Gas chromatography/mass spectrometry, OSC: 2, 3-Oxidosqualene cyclase, TcOSC1: *Taraxacum coreanum* multifunctional triterpene synthase; PCR: Polymerase chain reaction. qRT-PCR: quantitative real-time polymerase chain reaction

Declarations

Author contribution statement

Y.E.C designed the experiments and wrote the article. H.J.J constructed the transgenic tobacco. H.S.C performed genomic PCR and qPCR. J.Y.H performed the GC/MS analysis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

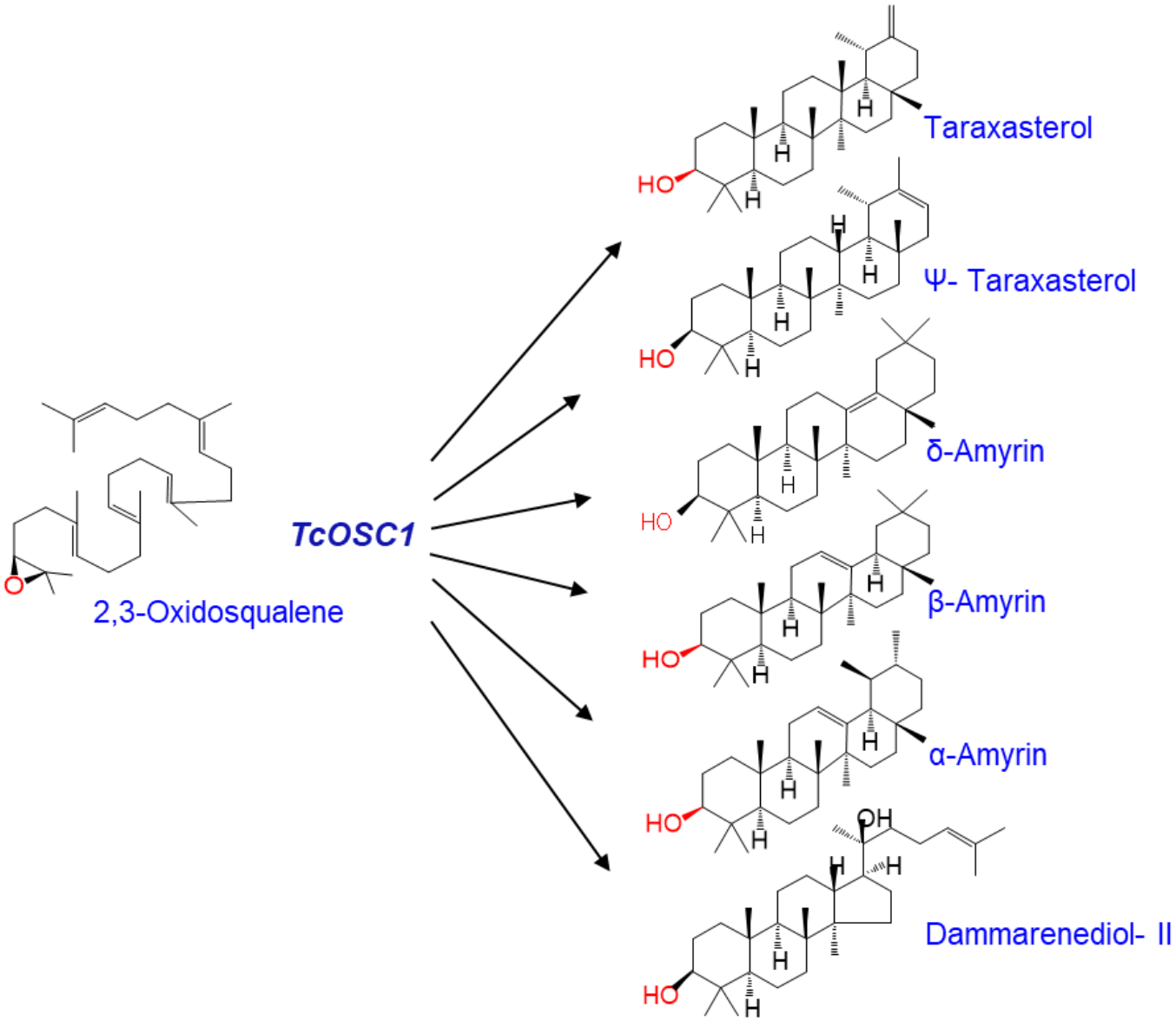

Figure 1

Biosynthesis of multiple triterpenes by T. coreanum TcOSC1 enzyme. 2,3-Oxidosqualene is converted to six triterpenes by TcOSC1 enzyme

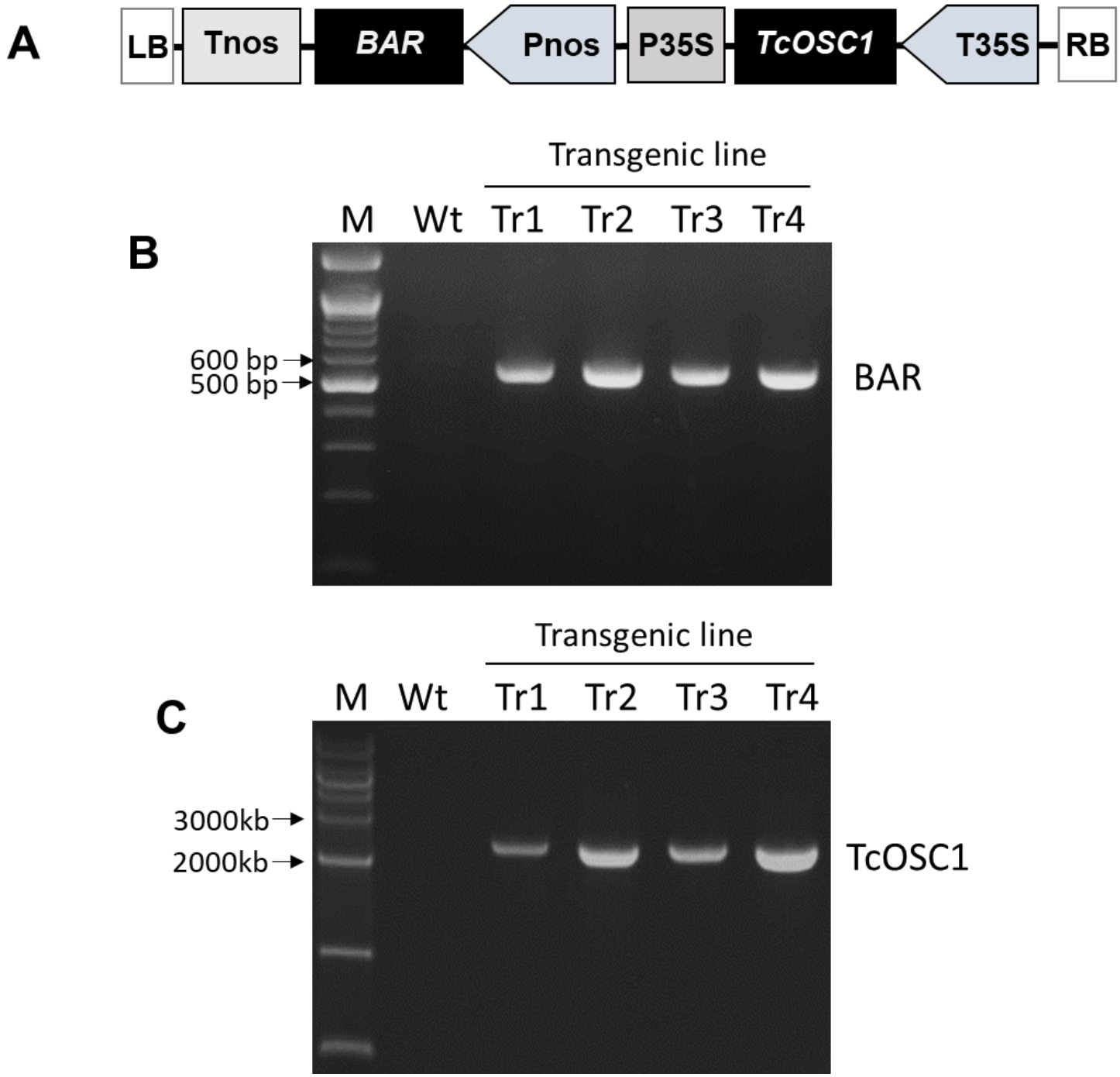

Figure 2

Vector construction for overexpression of TcOSC1 and detection of introduced genes in transgenic tobacco overexpressing TcOSC1. (A) T-DNA region of the plasmid for TcOSC1 gene overexpression. RB right T-DNA border, Tnos terminator region of the nopaline synthase gene, BAR gene encoding resistance to the herbicide BASTA, Pnos promoter region of the nopaline synthase gene, P35S CaMV 35S promoter

sequence, TcOSC1 cDNA sequences encoding the multifunctional triterpene synthase enzyme from T. coreanum, T35S CaMV 35S terminator sequence, LB left T-DNA border. (B) Confirmation of the introduced gene (BAR) by genomic PCR. (C) Confirmation of the introduced gene (TcOSC1) by genomic PCR.

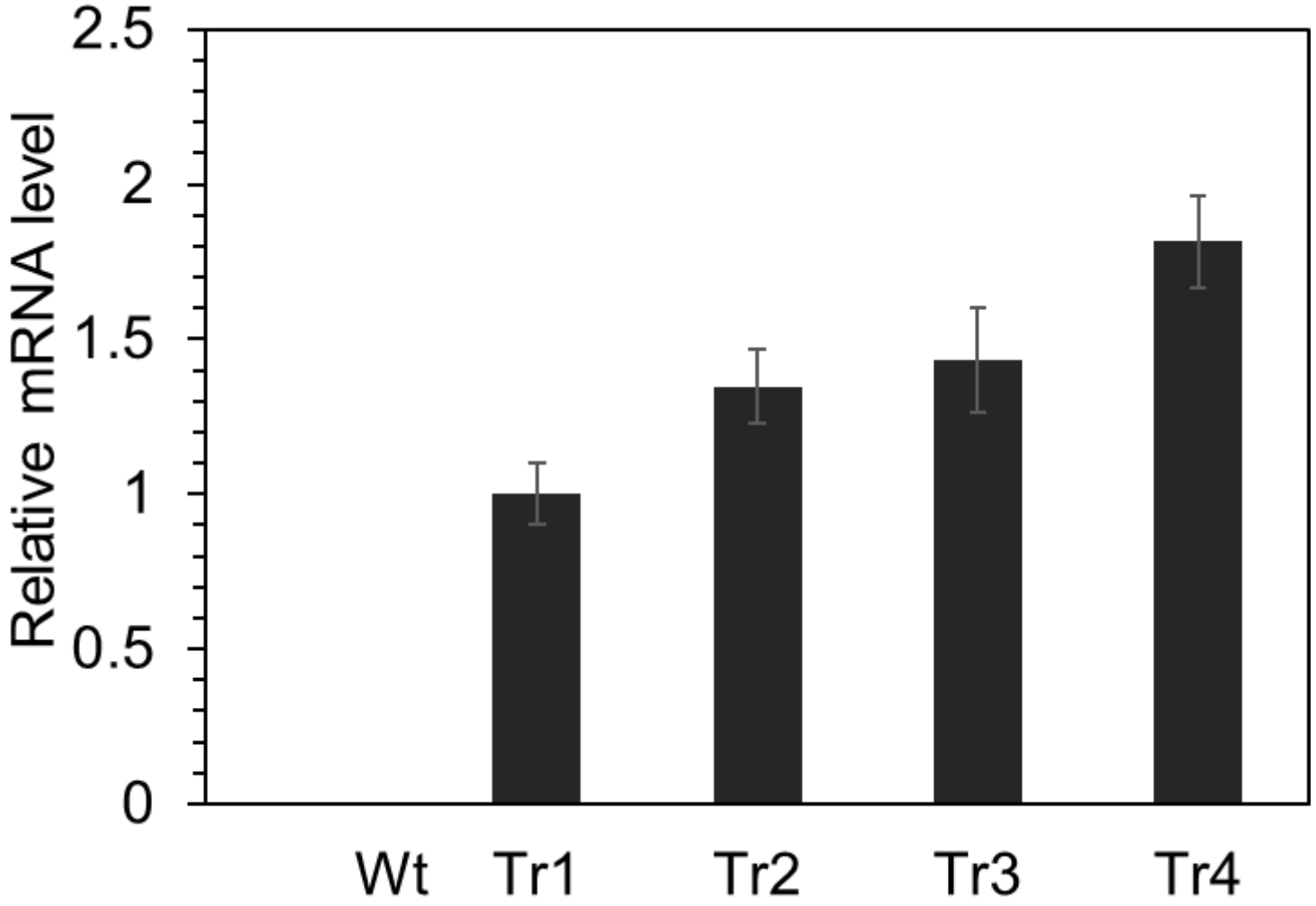

Figure 3

Expression of TcOSC1 in leaves of wild-type (Wt) plants and the transgenic lines (Tr1, Tr2, Tr3, and Tr4) by qRT-PCR. β-actin was used as a loading control. Data are mean values with the standard error obtained from three independent plants.

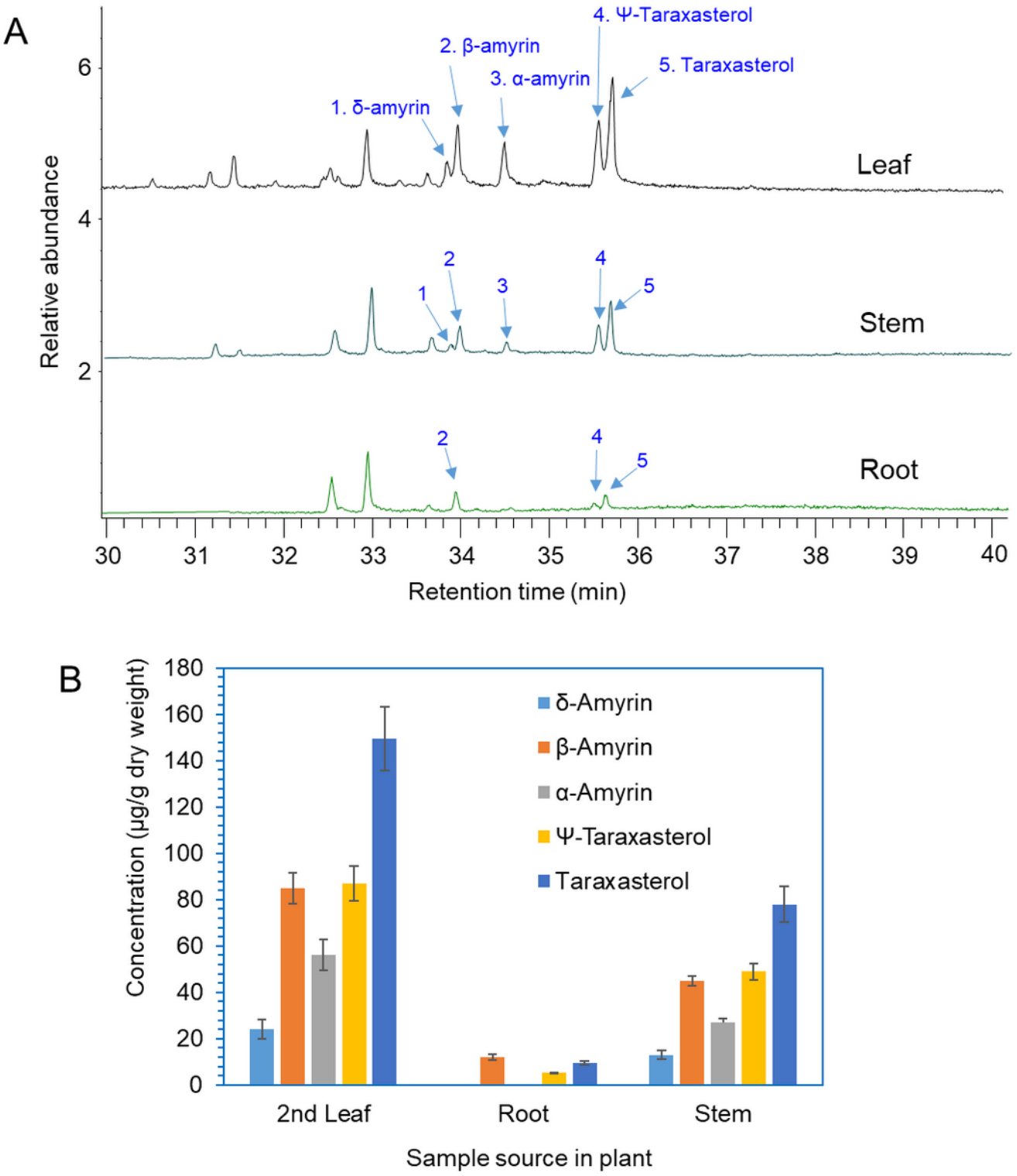

Figure 4

Triterpene analysis in different parts (leaf, stem, and root) of a line (Tr3) of transgenic tobacco overexpression of TcOSC1. (A) GC analyses of triterpenes in leaf, stem and root extracts from a Tr3 transgenic line. (B) Triterpene contents in leaf, stem and root extracts from a transgenic line (Tr3). Data are mean values with the standard error obtained from three independent plants.

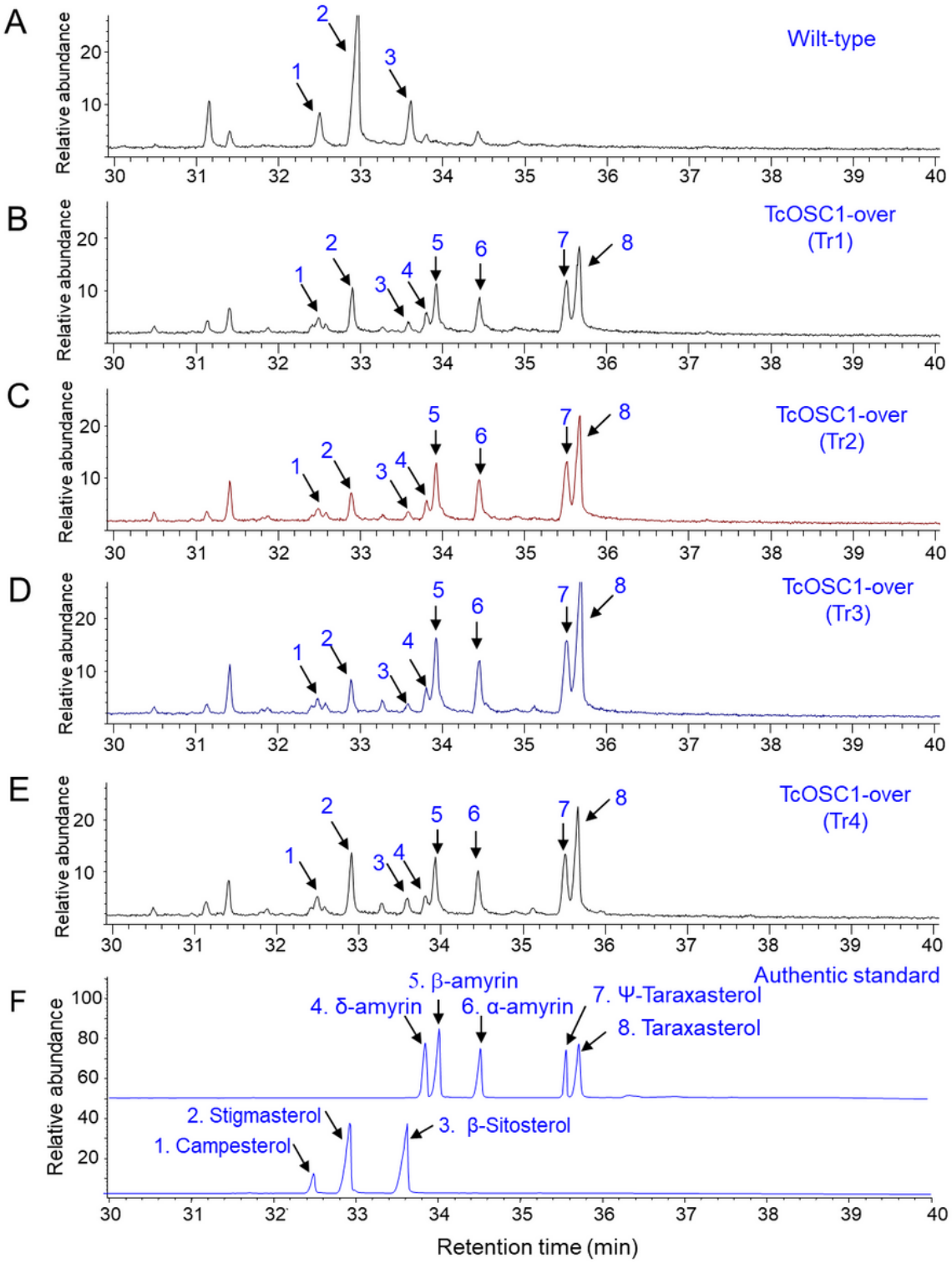

Figure 5

GC-MS analyses of triterpenes and phytosterols in the leaf extracts from wild-type and transgenic tobacco lines overexpressing TcOSC1. (A) GC chromatogram of triterpenes and phytosterols in leaf extracts of wild-type tobacco. (B-E) GC chromatogram of triterpenes and phytosterols in leaf extracts of transgenic tobacco lines (Tr1, Tr2, Tr3, and Tr4). (F) GC chromatogram of standard triterpenes and phytosterols.

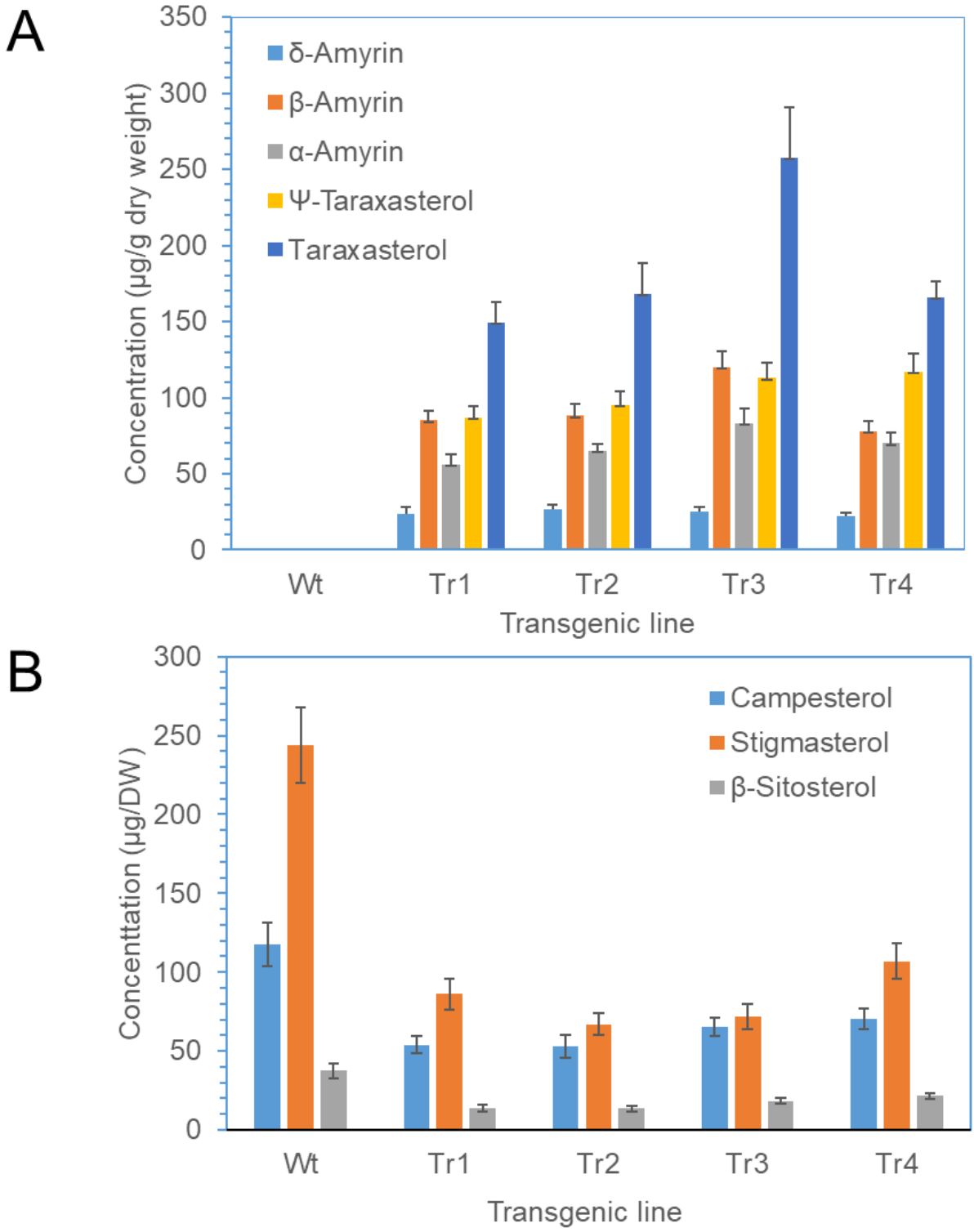

Figure 6

Triterpene and phytosterol contents in leaves of transgenic tobacco lines overexpressing TcOSC1. (A) Triterpene contents in leaves of four different transgenic lines. (B) Phytosterol (campesterol, β-sitosterol, and stigmasterol) contents in leaves of four different transgenic lines. Data are mean values with the standard error obtained from three independent plants.