Sugar availability modulates polyisoprenoid and phytosterol profiles in *Arabidopsis thaliana* hairy root culture

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Abstract

Sugars are recognized as signaling molecules regulating the biosynthesis of secondary metabolites in plants. Here, a modulatory effect of sugars on dolichol and phytosterol profiles was noted in the hairy roots of *Arabidopsis thaliana*. Arabidopsis roots contain a complex dolichol mixture comprising three groups ('families') of dolichols differing in the chain-length. These dolichols, especially the longest ones are accompanied by considerable amounts of polyprenols of the same length. The spectrum of polyisoprenoid alcohols, i.e. dolichols and polyprenols, was dependent on sugar type (glucose or sucrose) and its concentration in the medium. Among the long-chain dolichols Dol/Pren-20 (dolichol or prenol molecule composed of 20 isoprene residues) and Dol/Pren-23 were the main components at 0.5% and 2% glucose, respectively. Moreover, the ratio of polyprenols versus respective dolichols was also modulated by sugar in this group of polyisoprenoids, with polyprenols dominating at 3% sucrose and dolichols at 2% glucose. Glucose concentration affected the expression level of genes encoding *cis*-prenyltransferases, enzymes responsible for elongation of the polyisoprenoid chain.

The most abundant phytosterols of the *A. thaliana* roots, β-sitosterol, stigmasterol and campesterol, were accompanied by corresponding stanols and traces of brassicasterol, stigmast-4,22-dien-3-one and stigmast-4-en-3-one. Similarly to the polyisoprenoids, sterol profile responded to the sugar present in the medium, β-sitosterol dominating in roots grown on 3% or lower glucose concentrations and stigmasterol in 3% sucrose. These results indicate on involvement of sugar signaling in the regulation of *cis*-prenyltransferases and phytosterol pathway enzymes.

Short title: Sugars modulate polyisoprenoid and phytosterol profiles in Arabidopsis

Key words

dolichol; phytosterol; dolichyl ester; steryl ester; *cis*-prenyltransferase; *Arabidopsis thaliana*

Abbreviations

Dol-n – Dolichol composed of n isoprene residues; Pren – Polyprenol; CPT – *cis*-prenyltransferase;

1. Introduction

Terpenoids are involved in various cellular processes such as electron transport, photosynthesis, plant defense responses, hormonal regulation of development, and control of membrane fluidity [1]. Polyisoprenoids and phytosterols are representatives of this most numerous class of secondary metabolites [2,3].

Polyisoprenoid alcohols constitute a group of hydrophobic polymers occurring in almost all living organisms. These molecules consist of up to more than 100 isoprene residues (Fig. 1) with either a hydrogenated double bond in the α-residue (dolichols, syn. dihydropolyprenols) or an unsaturated one (polyprenols, syn. dehydrodolichols) [2]. Dolichols have been detected in mammalian and yeast cells and recently in plant roots [4,5]. An interesting feature of the polyisoprenoid alcohols of a given organism is their occurrence as a mixture commonly named ‘family’. The term ‘family’ denotes here a mixture with one dominant component and a Gaussian-like distribution of homologues. More complex mixtures containing two or three ‘families’ of polyprenols have been observed in plant photosynthetic tissues [2]. The polyisoprenoids occur mostly as free alcohols and esters with carboxylic acids, with only traces of phosphates [2]. Plant polyprenols are mainly esterified with acetic acid, although long-chain fatty acids (palmitic, oleic, linoleic, α-linolenic) have also been detected in some plant species [6,7]. The biological functions of free polyisoprenoid alcohols, i.e. dolichols and polyprenols in plants and other eukaryotes are largely unknown. They are postulated to act as modulators of properties of cellular membranes since studies on model membranes have shown that polyisoprenoids increase membrane fluidity and permeability [8-10]. On the other hand, the role of phosphorylated dolichols as cofactors in protein glycosylation and glycosylphosphoinositol (GPI) anchor synthesis in eukaryotic cells is well

characterized [11-13]. Recently, a new function for dolichols and polyprenols has been proposed - as a shield against reactive oxygen species (ROS) [14].

Sterols are crucial components found in eukaryotic cell membranes. They determine membrane fluidity and permeability [15]. What is more sterols are precursors of a wide range of bioactive compounds, e.g., brassinosteroid plant hormones taking part in vital cellular and developmental processes [16-18]. Sterols are found in cells in three forms: free sterols - the major form with a free 3β-hydroxyl group, steryl esters and steryl glucosides; no specific biological role has thus far been assigned to the two latter forms [13]. Plants, including *A. thaliana*, accumulate in their leaves various phytosterols with β-sitosterol, stigmasterol and campesterol (Fig. 1) as major components, and a small fraction of steryl esters [13]. There is no data on sterol composition of Arabidopsis roots.

All plant isoprenoids are synthesized from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) which are derived either from the cytoplasmic mevalonate (MVA) or the plastidial methylerythritol phosphate (MEP) pathways [for reviews see 19,20]. An initially formed intermediate - farnesyl diphosphate (FPP) - is further used to form numerous isoprenoid compounds. Elongation of FPP by an enzyme called *cis*-prenyltransferase (CPT) [21] results in the formation of polyprenyl diphosphate which is further converted to dolichol by polyprenol reductase [22]. Alternatively, condensation of two FPP molecules by squalene synthase results in the formation of squalene which is further converted to phytosterols by a set of dedicated enzymes [3].

While almost all the enzymatic steps of polyisoprenoid and phytosterol biosynthetic pathways have already been well described [3,5,19,20,21,22], the general mechanisms coordinating the isoprenoid metabolism with the cellular metabolic network are still a subject of extensive studies. One of the well recognized signaling molecules is glucose and hexokinase–dependent and hexokinase-independent signaling pathways are known to

participate in sugar sensing [23]. Very recently Pleiotropic Regulatory Locus 1 (PRL1) has been shown to integrate sugar responses with isoprenoid metabolism [24]. The signaling via PRL1 involves the regulation of the activity of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR1), the key enzyme of the MVA pathway, by its phosphorylation (causing inactivation) by SnRK1 kinase [25]. An involvement of regulatory promoter elements controlling gene expression in response to sugar has also been postulated [26].

Cultured plant tissues have proven to be good models for biochemical and molecular studies. Among others, hairy roots were found to be an abundant source of many classes of secondary metabolites [27] including dolichols considered as potential chemoterapeutics for patients suffering from shortage of dolichol phosphate (Congenital Disorder of Glycosylation type I) [22].

In this study a hairy root culture of *Arabidopsis thaliana* was characterized in terms of its content of polyisoprenoid alcohols and sterols and its modulation by type and concentration of sugar in the medium. Surprisingly, a complex, three-family pattern of dolichols was found in this tissue, which is the first example of such in higher Eukaryotes. The main sterol components were the same as found in the leaves. The carbon source and its availability modulated the profile of accumulated dolichols and sterols as well as the expression of genes encoding enzymes of their pathways. The results indicate the usefulness of the hairy root model for biochemical and molecular studies on polyisoprenoid and sterol metabolism in plants.

2. Materials and methods

2.1 Plant material and growth conditions

Plants of *Arabidopsis thaliana* ecotype Columbia 2n=10 were grown in sterile conditions on ½ Murashige and Skoog (MS) medium supplemented with vitamins (nicotinic acid, pyridoxine and thiamine) and inositol (5.6 mM) [28] at 20°C under 16/8 h photoperiod. The *Argobacterium rhizogenes* strain ATCC 15834 was used for transformation. Bacterial inoculum was grown overnight at 37^0C on solid LB medium. *A. thaliana* rosette leaves were cut into pieces and approx. 1 cm squares were incised using a scalpel and placed on the surface of ½ MS medium in a Petri dish. Then inoculum of *A. rhizogenes* was placed on the leaf surface and Petri dishes were sealed with parafilm and placed at 20^0C in darkness. After seven days the inoculated tissue was transferred twice to fresh ½ MS media supplemented with ampicillin, initially at a higher (500 mg/l) and then at a lower antibiotic concentration (300 mg/l), for 28 days in each case. Afterwards hairy roots from one explant were cut out and transferred to a fresh ½ MS liquid medium. Subsequently, hairy root cultures were transferred into a new medium (subcultured) every 21 days and grown in darkness at 22 °C on a rotary shaker at 105 rpm. After four subcultures, the hairy root culture was transferred to medium containing either sucrose (3%) or glucose (0.5, 1.0, 1.5, 2.0, 2.5, 3.0%) as the sole carbon source. In some experiments the growth of the culture was prolonged up to 42 days without medium change. Neither the root morphology nor sugar concentration in the medium changed significantly during the culture growth. When indicated, aliquots of roots were collected and frozen in liquid nitrogen for subsequent gene expression analysis and the remaining tissue was air-dried prior to lipid analysis.

2.2 Chemicals

All dolichol and polyprenol standards were from the Collection of Polyprenols (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw). Standards of sterols were from Sigma-Aldrich. Standards of fatty acid methyl ester mixture (37 species) and derivatizing chemicals for GC analysis were obtained from Supelco (Bellefonte, PA). Silica gel and RP-18 TLC plates and silica gel 60 for column chromatography were from Merck (Darmstadt, Germany), organic solvents (HPLC and p.a. grade) were from POCh (Gliwice, Poland). Murashige and Skoog Basal Salt Mixture and other chemicals were purchased from Sigma-Aldrich and were of analytical grade. RNeasy Plant Mini Kit was obtained from Qiagen (Hilden, Germany). DNase I, RNase-free, GeneRuler™ DNA Ladder and DNA Loading Dye were from Fermentas (Vilnius, Lithuania). SuperScript™ II First-Strand Synthesis System for RT-PCR and *Taq* DNA Polymerase were from Invitrogen (Carlsbad, CA).

2.3 Lipid extraction

Dry roots (10 g) were homogenized using a mortar and pestle. Lipids were extracted with 20 ml of acetone/hexane (1:1, by vol.) for 2 days at room temperature following the earlier-described procedure [4] with modifications. The extract was removed by decantation and the tissue was reextracted four times with new portions of the solvent mixture. All extracts were pooled and evaporated under a stream of nitrogen. The crude lipid fraction was divided into halves analyzed separately. Analysis of the total pool of lipids was performed after alkaline hydrolysis, thus one half was hydrolyzed (7.5% KOH in a mixture of water/toluene/ethanol, 1/6.6/5.5 by vol., containing 0.2% pyrogallol) at 95°C for 1 hour. Unsaponifiable lipids were extracted with hexane three times and purified on a silica gel 60 column. Purified lipids were analyzed by HPLC using suitable internal and external standards.

To analyze the content of isoprenoid esters, the second half of lipids was purified on a silica gel 60 column eluted with hexane containing increasing concentrations of diethyl ether (0–15%), to separate esters of polyisoprenoids and sterols and non-esterified forms of these compounds. The esters of polyisoprenoid alcohols and sterols were eluted with 1% diethyl ether in hexane, and free polyisoprenoids and sterols were eluted with 6% and 10% diethyl ether in hexane, respectively.

Native polyisoprenoid and steryl esters were then separated (silica gel 60 column eluted with linear gradient from 0% to 2% of diethyl ether in hexane) and split into halves**.** One portion of pure polyisoprenoid esters and steryl esters was used for estimation of carboxylic acid residues while the second half was subjected to alkaline hydrolysis and the liberated polyisoprenoids and sterols were used for estimation of the respective alcohols (see below). The recovery of the analytical procedure was approx. 85%. Each experiment was performed in triplicate and the presented data are means of three independent measurements.

2.4 HPLC/UV analysis of polyisoprenoids

Lipids were analyzed according to a previously described protocol [4] with modifications. Runs were performed on a 4.6 × 75 mm ZORBAX XDB-C18 (3.5 μm) reversed-phase column (Agilent, USA) using a Waters dual-pump apparatus, a Waters gradient programmer, and a Waters Photodiode Array Detector (spectrum range: 210-400 nm). The chain length and identity of lipids were confirmed by comparison with external standards of a polyprenol mixture (Pren-9, 11-23, 25) and a natural mixture of ram dolichols (from Dol-15 to Dol-24 with Dol-19 most abundant). Quantitative determination of polyisoprenoids and phytostereols was performed by using Dol-23, Pren-19 and cholestanol as internal and external standards. Integration of the HPLC/UV chromatograms was performed with the aid of the Empower (Waters) software.

2.5 HPLC/ESI-MS analysis of polyisoprenoids

HPLC/ESI-MS measurements were performed using an HP 1100 series HPLC system (Agilent Technologies) coupled to an API 365 triple quadrupole mass spectrometer (Applied Biosystems), as described previously [4].

2.6 Quantitative (qPCR) expression analysis of cis*-prenyltransferases (CPTs)*

Total RNA was extracted and purified using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were verified using a NanoDrop™ 1000 Spectrophotometer (THERMO Scientific, Waltham, MA). Before cDNA synthesis, RNA was treated with RNase-free DNase I (Fermentas) according to the manufacturer's instructions and then the first-strand cDNA synthesis was carried out with approximately 1.38 µg of RNA using the SuperScript™ II First-Strand Synthesis System for RT-PCR (Invitrogen) and oligo-dT primers according to the manufacturer's procedure. Six microliters of cDNA was used for real-time PCR using gene-specific primers in a total volume of 20 µl of Maxima™ SYBR Green qPCR Master Mix (Fermentas) in a Real-time thermal cycler PikoReal 96 (THERMO Scientific) according to the manufacturer's instructions. The cycle threshold (Ct) was used to determine the relative expression level of a given gene using the $2^{-\Delta\Delta Ct}$ method. The relative expression level of each gene was analyzed using PikoReal Software 2.0 (THERMO Scientific) after normalization with Actin (*ACT*) gene used as the internal reference. One-way ANOVA with Tukey's post test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA www.graphpad.com).

2.7 HPLC/UV analysis of sterols

Sterols were analyzed according to the previously described protocol with modifications [29]. Separation was performed on a Nova-Pack C18 column (3.9 × 300 mm, 4 μm; Waters, USA) using a Waters dual-pump apparatus, a Waters gradient programmer, and a Waters 2487 Dual λ Absorbance Detector. A mixture of methanol/acetonitrile, 3:7 (v/v), was used for isocratic elution at a flow rate of 1.2 ml/min. Detection wavelength was 205 nm. The identity of sterols was confirmed by comparison with external standards of stigmasterol, campesterol, β-sitosterol, β-sitostanol, ergosterol and cholesterol.

2.8 GC/FID analysis of sterols

Sterols were analyzed as trimethylsilyl (TMS) derivatives. To prepare the TMS derivatives, a sample of free sterols (4.5 mg) was supplemented with pyridine (250 μl) and a mixture of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% of trimethylchlorosilane (500 μl), kept at 70°C for 20 minutes in a tightly closed vial and then cooled at room temperature. The trimethylsililated sterols were analyzed by GC within 6 hours.

The GC apparatus (Agilent Technologies, type 7890A) was equipped with a split/splitless injector and a FID detector. A capillary HP-5 column (J & W Scientific Columns from Agilent Technologies) of 30 m length, 0.32 mm i.d. and 0.25 μm film thickness was used. Nitrogen, hydrogen and air flow-rates were maintained at 1 ml/min., 30 ml/min. and 400 ml/min., respectively. Inlet and detector temperature was kept at 250°C and 290°C, respectively, and the oven temperature was programmed as 65-230-280°C with a one-minute hold at 65°C, an increase rate of 20°C/min, a one-minute hold at 230°C, an increase rate of 8°C/min and 24-minute hold at 280°C. Sterols were identified by comparison with commercial standards.

2.9 GS/MS analysis of sterols

The GC-MS analysis was carried out on an Agilent 7890A gas chromatograph coupled to an Agilent 5975C MS Detector under electron impact ionization (70 eV). The MS scan range was 33-500 atomic mass units. The chromatographic column for the analysis was a DB-5ms capillary column (30 m, 0.25 mm, 0.25 μm). The carrier gas used was helium at a flow rate of 0.75 ml/min. Samples were analyzed with the column held at 265°C for 60 minutes. The injection was performed in the split mode (10:1) at 290°C. The identification of the compounds was achieved by comparison with NIST mass spectral library on the basis of the mass fragments and *m*/*z* values of each compound.

2.10 Synthesis of stigmast-4,22-dien-3-one and stigmast-4-en-3-one

Stigmasterol (41.2 mg, 0.1 mmol) or β-sitosterol (41.4 mg) were dissolved in 3 ml of dichloromethane and 32.3 mg (0.15 mmol) of pyridinium chlorochromate was added. The reaction mixture was stirred at room temperature for two hours. Progress of the oxidation reaction was analyzed on silica TLC plates in toluene:ethyl acetate (9:1, by vol.). To separate pyridinium chlorochromate from products, the reaction mixture was placed on a florisil column and elution with dichloromethane was performed. Further purification of the 3-oxo-steroids synthesized was performed on a silica gel 60 column eluted with increasing concentrations of diethyl ether in hexane (0%, 5%, 10%, 15%).

2.11 GC/FID analysis of fatty acids

Sterol esters (4.5 mg) were mixed with 1 ml of a 14% (w/w) solution of boron trifluoride in methanol. The vial was firmly closed and placed at 70°C. After 20 minutes the vial was cooled at room temperature and 1 ml of *n*-hexane and 1 ml of MilliQ water were added. Fatty acid methyl esters were extracted by vigorous shaking. The organic layer was collected and dried over anhydrous sodium sulfate and then analyzed by GC.

The GC apparatus (Agilent Technologies, 7890A) was equipped with a split/splitless injector, FID detector and capillary HP INNOWax column (30 m, 0.25 mm, 0.25 μm) (J & W Scientific Columns from Agilent Technologies). Nitrogen was used as the carrier gas at an average velocity of 30 ml / s. Hydrogen and air flow-rate was maintained at 30 ml / min and 400 ml / min, respectively. The inlet and detector temperature was kept at 250°C and 260°C, respectively, and the oven temperature was programmed as 50-220-260°C with a two-minute hold at 50°C, an increase rate of 4°C / min, 20-minute hold at 220°C, an increase rate of 20°C / min and a five-minute hold at 260°C.

The fatty acid methyl esters were identified by comparison of their retention times with those of commercially available FAMEs standards.

3. Results and discussion

3.1 Sugar modulates polyisoprenoid composition and content in hairy roots

A detailed study of polyisoprenoids accumulated in the hairy root culture of *A. thaliana* with the aid of HPLC/UV revealed three families of homologous polyisoprenoid alcohols differing in chain length (Fig.2A). The three families comprised short-chain dolichols, from Dol-12 to -14 (dolichol molecules composed of 12 up to 14 isoprene residues), with the most abundant component Dol-13, medium-chain dolichols with Dol-16 most abundant, and long-chain ones, from Dol-19 to Dol-30, with Dol-20 dominant. The dolichols were accompanied by various amounts of corresponding polyprenols (α-unsaturated dolichol counterparts).

The identity of dolichols isolated from Arabidopsis roots purified after alkaline hydrolysis (see Materials and methods) was unambiguously confirmed by HPLC/ESI-MS. The main component of the dolichol mixture, Dol-16 with the molecular formula $C_{80}H_{132}O$, gave a pseudomolecular ion peak m/z 1132.3 ($[M+Na]^+$), while Dol-20 ($C_{100}H_{164}O$) and Dol-23

($C_{115}H_{188}O$) gave pseudomolecular ion peaks m/z 1404.5 ($[M+Na]^+$) and 1608.7 ($[M+Na]^+$), respectively. The molecular masses of other prenologues (from C_{65} to C_{130}) were also confirmed by HPLC/ESI-MS (see Supplemental Table 1).

Such complexity of the dolichol pattern was rather unexpected since only one dolichol family comprised of 6-8 members has been detected in all plant and animal tissues studied before. The only exception was the yeast *S.cerevisiae* where, upon aging or starvation (for glucose or nitrogen), a second family of longer dolichols (Dol-19 to-23, Dol-21 most abundant) had been reported in addition to the typical dolichol mixture (Dol-14 to -18, Dol-16 most abundant) [30].

A comparison of the polyisoprenoids derived from cultures grown under various conditions (Table 2) showed that the composition of the long-chain ones varied with the concentration and type of sugar present in the growth medium. In the medium containing 3% sucrose Dol/Pren-21 were the most abundant, while Dol/Pren-20 and Dol/Pren-23 dominated in roots grown on 0.5% and 2.0% glucose, respectively (Table 2). What is even more interesting, a careful inspection of the HPLC/UV chromatogram revealed the presence of much longer dolichols (up to Dol-35) exclusively in roots grown on 1%, 1.5% or 2% glucose.

We also noticed that the carbon source and its concentration affected the dolichol:polyprenol ratio in the long-chain family of polyisoprenoid alcohols. The Dol:Pren ratio (as calculated from roughly integrated HPLC chromatograms) was approximately 0.8/1 in the roots grown on 3% sucrose versus 2.5/1 in the cultures from 2% glucose (Fig. 2B). That result suggested that the type of sugar and its abundance could affect the activity of enzyme(s) responsible for conversion of polyprenols into dolichols. This phenomenon needs further studies of which identification and characterization of the polyprenol reductase(s) involved is the first indispensable step. The occurrence of a polyprenyl reductase (SRD5A3-like gene product) in

Arabidopsis leaves has been reported recently [31] while its human counterpart had been characterized only one year earlier [22].

The total polyisoprenoid content in roots was also dependent on the carbon source and its concentration (Fig. 3). The highest accumulation of polyisoprenoid alcohols occurred in roots grown in the medium containing 3% sucrose (65.5 μg/g dry tissue) while roots grown on glucose showed lower accumulation (from 22.5 to 32.4 μg/g dry tissue, depending on the sugar concentration).

Numerous studies have reported on the accumulation of polyisoprenoids in various plant and animal tissues [2], but little is known on the regulation their synthesis. The mechanism of the concomitant regulation of genes encoding putative CPTs, enzymes elongating the polyisoprenoid chain, has not been elucidated yet.

3.2 Influence of carbon source on the expression of cis*-prenyltransferases*

As discussed above, the type of sugar and its concentration in the culture medium affected the composition of accumulated dolichols. This observation prompted us to investigate the effect of sugar on the expression of genes encoding CPTs. As many as ten genes encoding putative CPTs have been identified in the Arabidopsis genome but only three AtCPT have been characterized at the molecular level [32-35].

Six genes encoding putative CPTs were found to be expressed in Arabidopsis roots [21] and their individual expression levels were quantified by qPCR employing specific primers (Table 1). Variable, although statistically significant effects of glucose and sucrose concentrations on the transcript level were observed for all the genes studied (*AtCPT1*, *AtCPT2*, *AtCPT3*, *AtCPT6*, *AtCPT7* and *AtCPT9*) (Fig. 4). *CPT3* and *CPT7* were induced the most by higher glucose concentrations while *CPT6* and *CPT9* by sucrose. For *CPT1* and *CPT2* a weak induction was noted at high glucose or sucrose.

That diverse stimulatory effect of sugars on the individual CPTs suggests that the complex regulatory system responsible for polyisoprenoid biosynthesis is adjustable to the changing environmental conditions. It is not clear why Arabidopsis should need ten CPT isoenzymes and in fact they diverse response to sugar reported here suggests that they could be dedicated to responding to various environmental stimuli. If they show different preferences towards different substrates and/or catalyze the synthesis of products of different chain length this could be a mechanism responsible for adjusting the spectrum of polyisoprenoids to environmental signals. This hypothesis requires further detailed studies. Moreover, with only three CPTs characterized it is difficult to speculate on their individual functions and possible redundancy.

3.3 Influence of culture growth on dolichol accumulation

We checked the time-course content of accumulation of polyisoprenoid alcohols in the hairy root tissue during the culture growth in the medium containing 2% glucose (Fig. 5). The composition of the polyisoprenoids was constant during the course of this experiment (data not shown) while their overall content fluctuated modestly: after 21 days it decreased to 32.5 µg/g d.w. (approx. 74% of the initial value) to increase again to 57.8 µg/g d.w. (approx. 132% of the starting material) at the forty-second day of growth. An increase of the polyisoprenoid content during the life-span has been observed in all tissues studied [2]. The initial decrease of the dolichol content observed here most probably reflects the physiological status of growing roots since the whole root bundle is taken for analysis. The starting material – the root inoculum – is a mature root tissue with a high dolichol content. During the first two-three weeks of the culture growth new roots are appearing ubiquitously and they exhibit the low dolichol content typical for young tissue (Skorupinska-Tudek et al., unpublished). On the other hand, it is also plausible that degradation of dolichol is involved in determining its

cellular concentration. The mechanism of dolichol catabolism remains unknown even though its half life has been estimated in animal tissue ($t_{½}$ 40-70 h and 80-140 h in sea urchin embryo and rat liver, respectively [36,37]), and oxidative breakdown via conversion to aldehyde [38,39] or carboxylic acid [40,41] has been postulated.

3.4 Structure of dolichyl esters accumulated in Arabidopsis roots - HPLC/ESI-MS analysis

The polyisoprenoid alcohols (predominantly dolichols) accumulated in the *Arabidopsis thaliana* hairy roots occurred as esters of short-chain carboxylic acids, mainly propionic acid, and no free dolichols were detected at any time-point studied. The dolichyl esters were initially analyzed by TLC and HPLC/UV and a more detailed analysis was carried out by HPLC/ESI-MS. The R_f values and retention times of esterified dolichols from *A. thaliana* were compared with standards of dolichyl propionates and acetates obtained by acylation of a dolichol standard (Dol-19 acetate, propionate and palmitate) and re-acylation of the isolated mixture of native Arabidopsis dolichols (Supplemental Fig. 1). Finally, the identity of the dolichyl esters was confirmed by HPLC/ESI-MS. The most abundant compound Dol-16 propionate with the molecular formula $C_{83}H_{136}O_2$ gave a pseudomolecular ion ($[M+Na]^+$) peak *m/z* 1188.0. The molecular masses of all other esterified homologues were also in agreement with the dolichyl propionate structure.

3.5 Free sterols accumulated in Arabidopsis roots - GC-FID and GC-MS analysis

Free sterols and steryl esters were isolated and characterized separately.

A GC-FID chromatogram of free sterols isolated from hairy roots of Arabidopsis and analyzed as trimethylsilyl ethers indicated (Fig. 6) that stigmasterol, β-sitosterol, and campesterol were the main accumulated sterols, with accompanying stanols. In order to further characterize the fraction of free sterols, GC-MS was performed. It fully confirmed the

above results and gave additional information indicating the presence of small amounts of brassicasterol, stigmast-4,22-dien-3-one and stigmast-4-en-3-one (Fig. 1, Supplemental Fig. 2).

Quantitative estimation of free sterols revealed that the most abundant one – stigmasterol - constituted 43.4% of all sterols (Table 3) in roots grown on 3% sucrose.

Benveniste reported a typical plant sterol profile for wild-type *Arabidopsis thaliana* ecotype Columbia leaves comprising β-sitosterol (64% of sterol pool), campesterol (11%), stigmasterol (6%), isofucosterol (3%), and brassicasterol (2%) [3]. Although the sterol composition of the Arabidopsis hairy roots turned out to be clearly different to that of the leaves, three sterols - β-sitosterol, campesterol and stigmasterol in different proportions - are dominant in both organs.

3.6 Esterified sterols accumulated in Arabidopsis roots - GC-FID identification of sterols and fatty acids

In parallel to free sterols, the fraction of steryl esters with carboxylic acids was also analyzed. Native steryl esters were hydrolyzed and analyzed by means of GC-FID as described above. The profile of sterol esters was very much like that of free sterols and comprised β-sitosterol, stigmasterol, campesterol, brassicasterol, sitostanol, stigmastanol and ergostanol (Fig. 6). Interestingly, in this case β-sitosterol was the dominant component of the sterol mixture from roots grown on 3% sucrose, in contrast to the free sterols (Table 3). The sterols were esterified with a wide range of fatty acids (Table 4, Supplemental Fig. 3.). The most abundant acyl residues, palmitic (C16:0), oleinic (C18:1n9c), linolenic (C18:2n6c) and α-linolenic (C18:3n3) were accompanied by butyric (C4:0), lauric (C12:0), myristic (C14:0), pentadecanoic (C15:0), palmitoleic (C16:1), heptadecanoic (C17:0), stearic (C18:0), arachidic (C20:0), behenic (C22:0), lignoceric (C24:0) and nervonic (C24:1n9) (Table 4). The

composition of steryl esters in Arabidopsis roots is similar to that of tobacco and many other plant leaves [42,43].

3.7 Sugar modulates sterol composition and content in A. thaliana *hairy roots*

Analysis of the effect of glucose on the profile of sterols revealed that similarly to polyisoprenoids also the sterol composition was modulated by glucose concentration. Interestingly, β-sitosterol was most abundant in roots grown on 3% glucose while stigmasterol was the dominant component of the total sterol mixture isolated from Arabidopsis hairy roots grown on 0.5% or 2% glucose, or 3% sucrose (Table 5). Similarly to polyisoprenoids, the content of sterols was the highest in roots grown on 3% sucrose.

The observed shift of the sterol profile in response to changed glucose concentration suggests the existence of a yet unknown mechanism regulated by glucose signal(s) modulating sterol biosynthesis in roots.

4. Conclusions

Elucidation of the mechanisms responsible for regulation of the isoprenoid biosynthesis pathways is in the focus of many research groups. Heterotrophically grown hairy root culture of *A. thaliana* is an interesting model for such studies however data obtained hitherto require further confirmation in the *in vivo* system. Elucidation of the polyisoprenoid and sterol profiles described here provides a valuable stepping stone for further experiments. Moreover, this report suggests an influence of glucose signal(s) on isoprenoid metabolism, both polyisoprenoids and sterols. Glucose availability modulated not only the profile and content of the isoprenoids, but also the transcription of genes for the crucial enzymes of polyisoprenoid biosynthesis, *cis*-prenyltransferases.

Saccharides are known to exert a double effect on the plant cell metabolism. On the one hand glucose, sucrose and other saccharides are readily metabolizable carbon sources which, upon glycolysis, provide intermediates further used by the cell's metabolic pathways, e.g., the isoprenoid-producing MEP (glyceraldehyde 3-phosphate and pyruvate) and MVA (acetate) pathways [19,20]. On the other hand, glucose is also a signaling molecule. The mechanism of glucose signaling in the plant metabolism has been studied extensively and various signaling pathways have been suggested [23,24,25,26].

Despite the growing body of data on the regulatory mechanisms involved, these transcriptional and post-transcriptional networks are still not fully understood. The suggested application of dolichols as drugs supplementing the cellular dolichol pool in patients with Congenital Disorder of Glycosylation type 1 [22] draws further attention to the mechanisms regulating their biosynthesis.

Acknowledgements

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Supplementary data

Supplementary data to this article can be found online

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Figure legends

Fig. 1 Structures of polyisoprenoid alcohols (dolichol, polyprenol) and phytosterols found in *A. thaliana* hairy roots grown *in vitro*. t (=2) and c (> 8) stand for internal *trans* and *cis* while α and ω – for OH- and C-terminal isoprene residue, respectively.

Fig. 2 Composition of polyisoprenoids extracted from hairy roots of *Arabidopsis thaliana*. The complex mixture comprises three families of short-, medium- and long-chain polyisoprenoids; the most abundant compounds of each family are indicated. A) HPLC/UV chromatogram of polyisoprenoids extracted from roots grown on 3% sucrose; B) The pattern of dolichols and polyprenols is changed when roots are grown on various concentrations of glucose or sucrose. Shown are expanded regions of HPLC chromatograms containing long-chain dolichols isolated from roots grown on media containing 2% glucose or 3% sucrose. The results were confirmed in three independent biological samples.

Fig. 3 Polyisoprenoid alcohols of roots cultured on different sugar types and concentration. Arabidopsis roots were cultivated for 21 days on media with various concentrations of sugars, dolichols were estimated by HPLC/UV as described in Materials and methods. Values (±SD) represent means of three independent experiments.

Fig. 4 Effect of sugars on expression of genes encoding *cis*-prenyltransferases isoenzymes. Hairy roots were grown on media containing various concentrations of sugars. Relative levels of expression were measured by qPCR using Actin2 as reference, with expression in 0.5% glucose set at 1. Data are mean values of three independent measurements, error bars are indicated. *P* value was determined by one-way ANOVA with Tukey's post test.

Fig. 5 Effect of hairy root culture growth on the content of polyisoprenoid alcohols. Roots were grown on 2% glucose for indicated time, dolichols were estimated by HPLC/UV as described in Materials and methods. Each bar represents the mean value of three independent experiments. Error bars are indicated.

Fig. 6 Composition of free sterols and steryl esters in hairy roots. GC/FID chromatograms of sterols isolated as free alcohols (top) and esters (bottom) from roots grown on 3% sucrose. Representative chromatograms out of three for independent biological samples are shown.

Table 1

Table 1. Primers used in qPCR to evaluate expression of *Arabidopsis thaliana* *cis*-prenyltransferases

Name of Arabidopsis CPT	Locus Tag	Primer sequence
AtCPT1	At2g23410	F: 5'-GTGGCAACTTGCTTATTCCG-3' R: 5'-CCTACGCTGATACGAAGC-3'
AtCPT2	At2g23400	F: 5'-TTGTCCGAGAGGAGGAGCTAC-3' R: 5'-TGCCGTCGTCAATCCGTCTC-3'
AtCPT3	At2g17570	F: 5'-GCGCTTATGTCGATGCTG-3' R: 5'-CAGACTCAACCTCCTCAGG-3'
AtCPT6	At5g58780	F: 5'-GACGATTATGACAACGAGCAAC-3' R: 5'-ATGTCTTGGCATCAGCTCTC-3'
AtCPT7	At5g58770	F: 5'-TATCTCTACGAGTTCCTACTCC-3' R: 5'-CTACTTAACCGCCATCGC-3'
AtCPT9	At5g58784	F: 5'-AGCATGTGGCGGTTATATTGG-3' R: 5'-TTCTCCATGAGCCTTCTCG-3'
Actin2	At3g18780	F: 5'-GACCAGCTCTTCCATCGAGAA-3' R: 5'-CAAACGAGGGCTGGAACAAG-3'

Table 2

Table 2. Dolichol spectrum in Arabidopsis hairy roots – effect of carbon source

Numbers in bold indicate dominating prenologues.

Carbon source		Dolichols (number of isoprene units)																							
Glucose	0.5%	12	**13**	14	15	**16**	17	18	19	**20**	21	22	23	24	25	26	27	28	29	30					
	1.0%	12	**13**	14	15	**16**	17	18	19	**20**	21	22	**23**	24	25	26	27	28	29	30	31	32	33	34	35
	1.5%	12	**13**	14	15	**16**	17	18	19	**20**	21	22	**23**	24	25	26	27	28	29	30	31	32	33	34	35
	2.0%	12	**13**	14	15	**16**	17	18	19	20	21	22	**23**	24	25	26	27	28	29	30	31	32	33	34	35
	2.5%	12	**13**	14	15	**16**	17	18	19	20	**21**	22	23	24	25	26	27	28	29	30					
	3.0%	12	**13**	14	15	**16**	17	18	19	20	**21**	22	23	24	25	26	27	28	29	30					
Sucrose	3.0%	12	**13**	14	15	**16**	17	18	19	20	**21**	22	23	24	25	26	27	28	29	30					

Table 3

Table 3. Composition of sterols accumulated as free alcohols and esters in hairy roots of *A. thaliana* grown on 3% sucrose. Data are obtained from GC/MS estimation (see Materials and methods).

Sterol	Sterol fraction			
	Free sterol		Esterified sterol	
	µg/g d.w.	%	µg/g d.w.	%
Total	2234	100	81	100
Brassicasterol	31.3	1.4	3.3	4.1
Campesterol	375.3	16.8	13.4	16.6
Campestanol	35.7	1.6	2.3	2.8
Stigmasterol	969.6	43.4	19.4	24.0
β-Sitosterol	661.3	29.6	36.7	45.3
Stigmastanol	71.5	3.2	5.9	7.2
Stigmast-4,22-dien-3-one	46.9	2.1	n.d.*	0
Stigmast-4-en-3-one	42.4	1.9	n.d.*	0

* n.d., not detected

Table 4

Table 4. Fatty acid composition of steryl esters isolated from *A. thaliana* hairy roots grown on 3% sucrose

Fatty acid	Retention time [min]	% of total FA
C4:0	4.63	0.18
C12:0	27.86	0.49
C14:0	33.52	0.98
C15:0	36.17	0.64
C16:0	38.80	16.47
C16:1	39.40	2.10
C17:0	41.15	0.37
C18:0	43.53	2.28
C18:1n9c	44.06	12.89
C19:2n6c	45.25	37.61
C18:3n3	46.88	16.70
C20:0	48.58	0.54
C22:0	56.28	1.86
C24:0	66.58	1.33
C24:1n9	67.58	5.56

Table 5

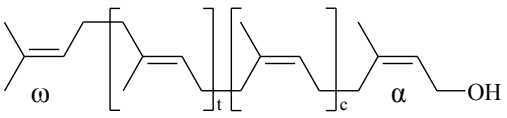
Table 5. Effect of sugar type and concentration on sterol content and profile (shown as ratio of two dominating sterols) in *A.thaliana* hairy roots. Content of sterols was estimated using HPLC/UV (see Materials and methods).

Sugar concentration	Stigmasterol	β-Sitosterol	Total sterol content [µg/g d.w.]
0.5% glucose	1.4	1	2129±234
2.0% glucose	1.3	1	2048±234
3.0% glucose	0.7	1	1476±126
3.0% sucrose	1.5	1	2234±268

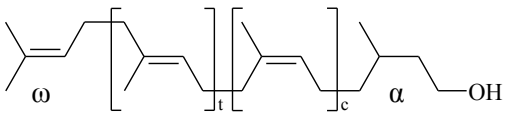
Figure 1

Fig. 1

A

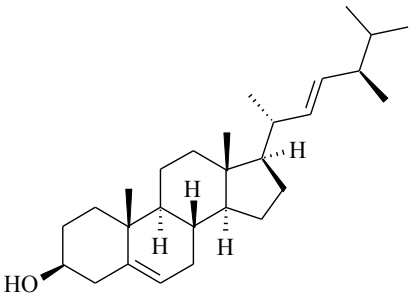


Polyprenol

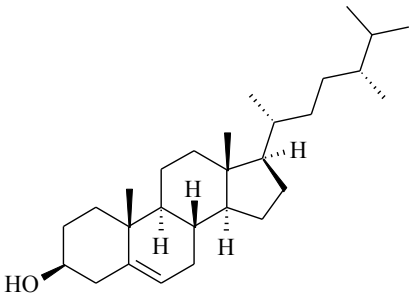


Dolichol

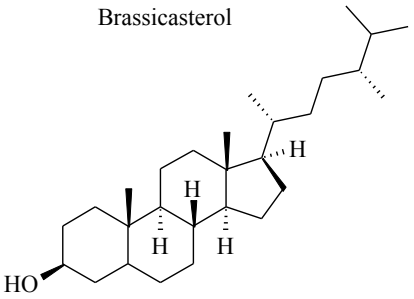
B



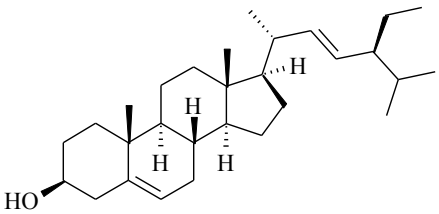
Brassicasterol



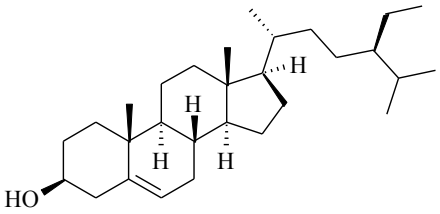
Campesterol



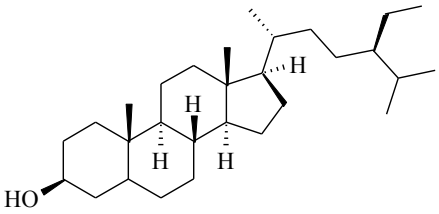
Campestanol



Stigmasterol



Sitosterol



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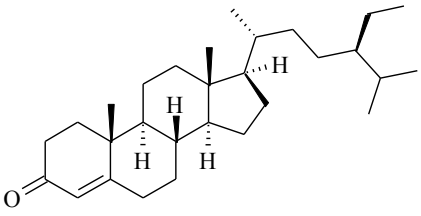
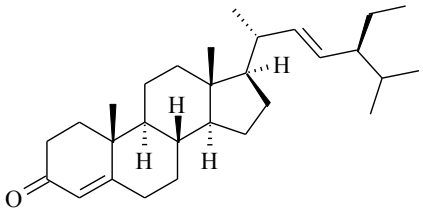


Stigmast-4-en-3-one

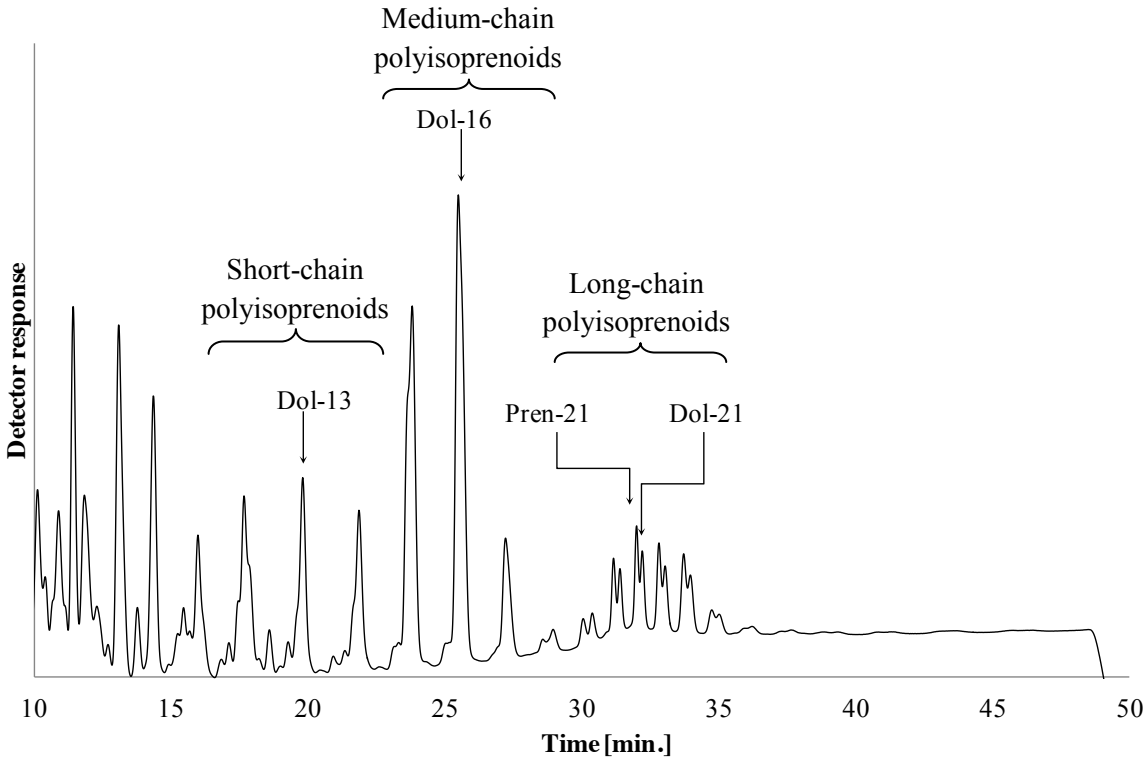


Stigmast-4,22-dien-3-one

Figure 2

Fig. 2

A



B

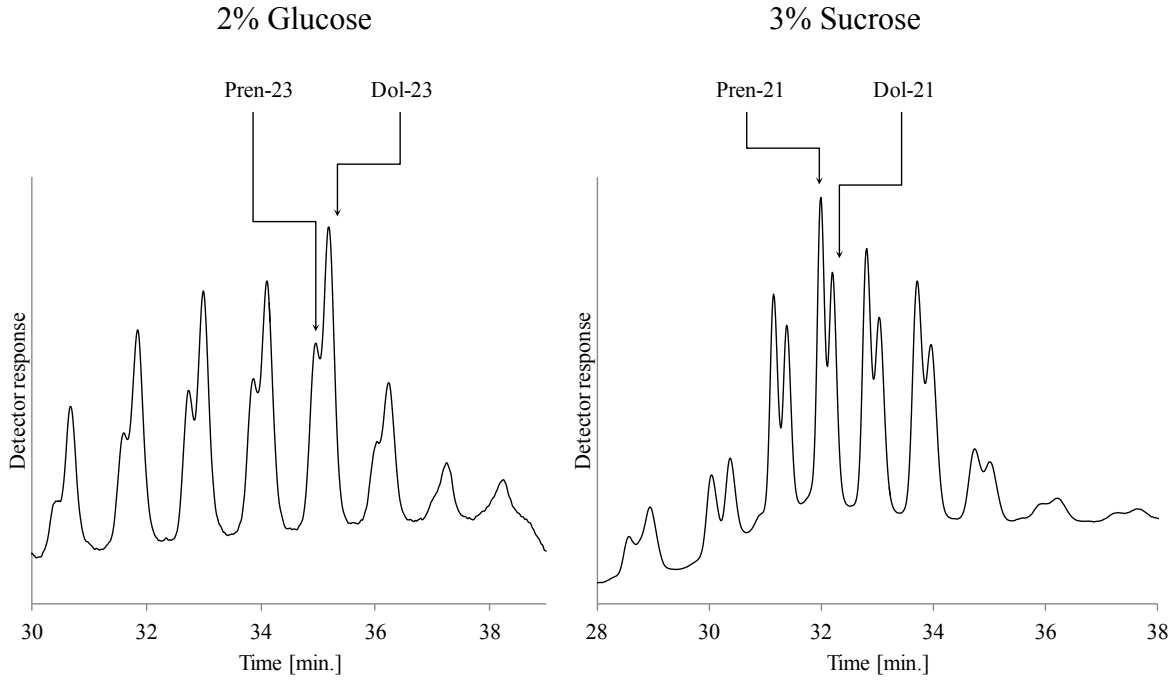


Figure 3

Fig. 3

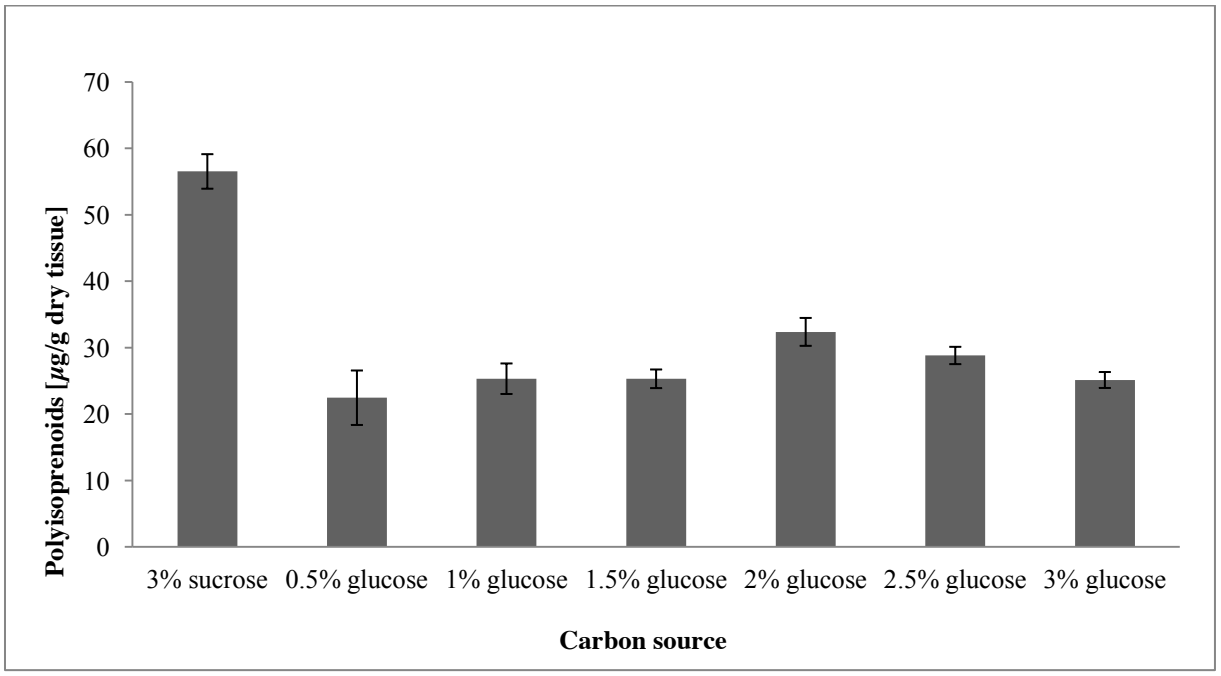


Figure 4

Fig. 4

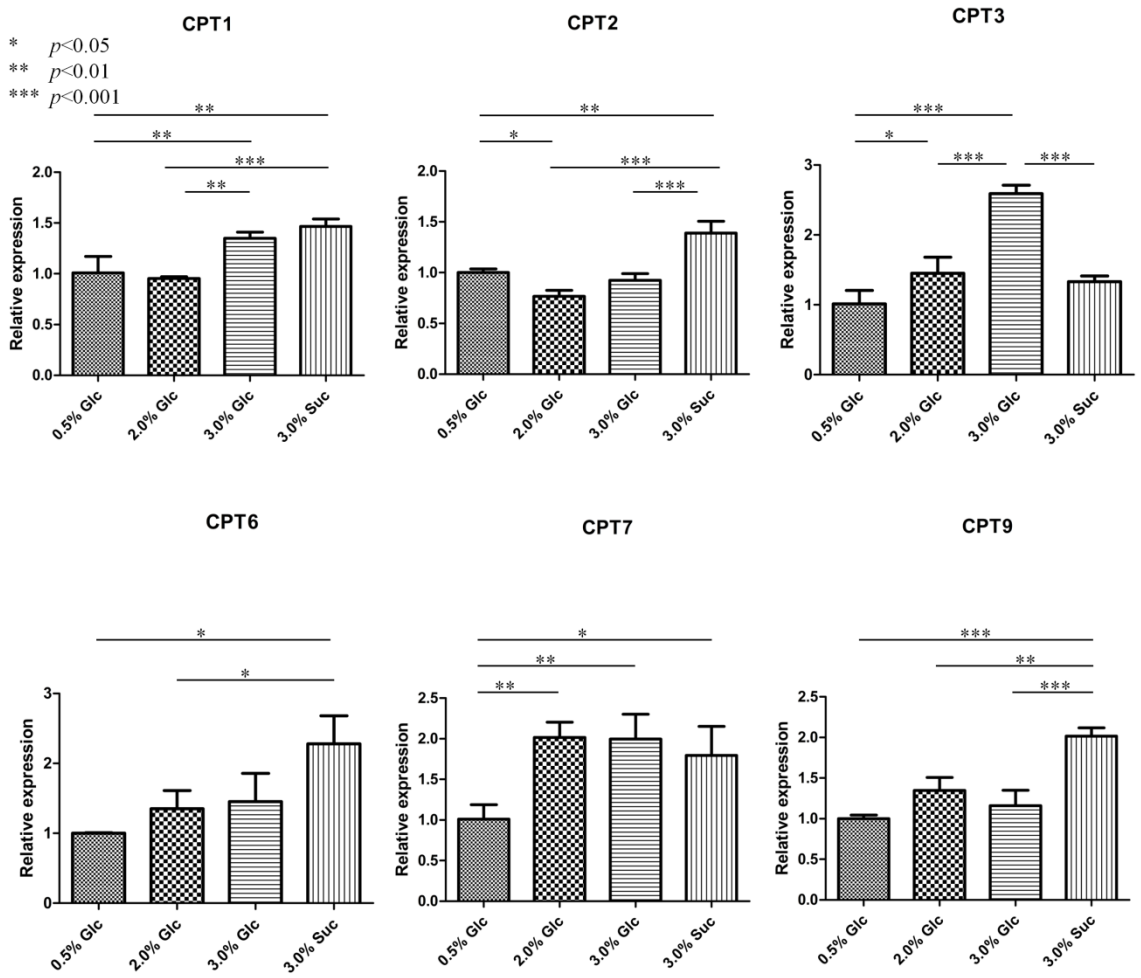


Figure 5

Fig. 5

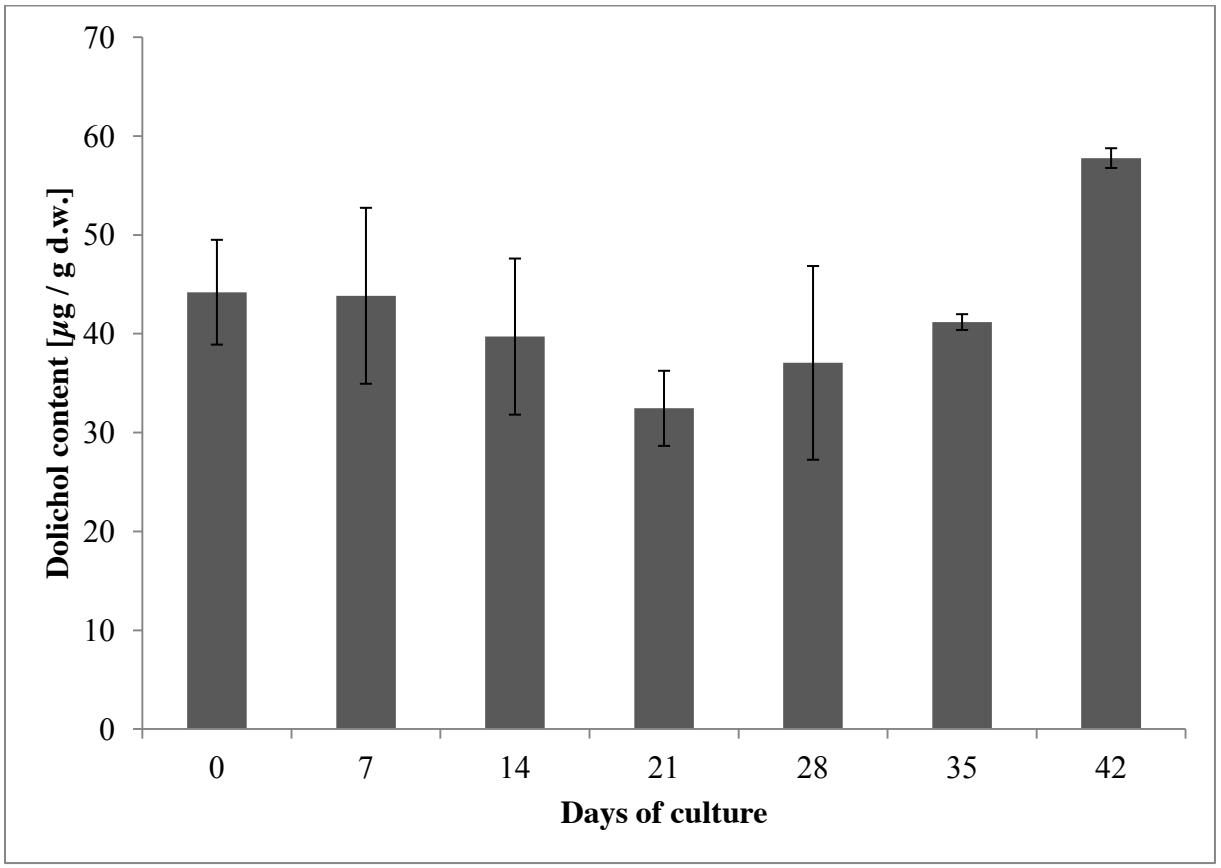


Figure 6

Fig. 6