Plant Oxidosqualene Metabolism: Cycloartenol Synthase–Dependent Sterol Biosynthesis in *Nicotiana benthamiana*

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**Abstract**

The plant sterol pathway exhibits a major biosynthetic difference as compared with that of metazoans. The committed sterol precursor is the pentacyclic cycloartenol (9β,19-cycloolanost-24-en-3β-ol) and not lanosterol (lanosta-8,24-dien-3β-ol), as it was shown in the late sixties. However, plant genome mining over the last years revealed the general presence of lanosterol syntheses encoding sequences (LAS1) in the oxidosqualene cyclase repertoire, in addition to cycloartenol syntheses (CAS1) and to non-steroidal triterpene syntheses that contribute to the metabolic diversity of C₅₀H₉₀O compounds on earth. Furthermore, plant LAS1 proteins have been unambiguously identified by peptidic signatures and by their capacity to complement the yeast lanosterol synthase deficiency. A dual pathway for the synthesis of sterols through lanosterol and cycloartenol was reported in the model *Arabidopsis thaliana*, though the contribution of a lanosterol pathway to the production of 24-alkyl-Δ⁵-sterols was quite marginal (Ohyama et al. (2009) PNAS 106, 725). To investigate further the physiological relevance of CAS1 and LAS1 genes in plants, we have silenced their expression in *Nicotiana benthamiana*. We used virus induced gene silencing (VIGS) based on gene specific sequences from a *Nicotiana tabacum* CAS1 or derived from the solgenomics initiative (http://solgenomics.net/) to challenge the respective roles of CAS1 and LAS1. In this report, we show a CAS1-specific functional sterol pathway in engineered yeast, and a strict dependence on CAS1 of tobacco sterol biosynthesis.

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**Introduction**

Plants, algae and some protists synthesize their sterols through a biosynthetic route that contains a pentacyclic steroidal cyclization product of 2,3-oxidosqualene, namely, cycloartenol (9β,19-cycloolanost-24-en-3β-ol, Fig. 1) the product of the cycloartenol synthase (CAS1, EC 5.4.99.8) [1–5]. In contrast, other organisms like mammals and fungi use lanosterol (lanosta-8,24-dien-3β-ol, Fig. 1), an isomeric tetracyclic steroidal cyclization product of 2,3-oxidosqualene that is made by the lanosterol synthase (LAS1, EC 5.4.99.7). The post-squalene biosynthetic pathways leading to Δ⁵-sterols from cycloartenol or from lanosterol have been described comprehensively with respect to the enzymes implicated and to the chemistry of the reactions considered [6–8]. However, the rationale of the conserved biosynthetic detour through 9β,19-cyclopropyl sterol intermediates in plants, algae and protists is not understood [9]. Higher plants have in addition the capacity to produce a huge array of mostly dispensable non steroidal 2,3-oxidosqualene cyclization products (for instance β-amyrin, Fig. 1) generated by various triterpene synthases that have been classified into multiple families [10,11]. The distribution of a representative set of oxidosqualene cyclases (OSCs) in a phylogenetic tree gives a clear view of main groups (Fig. 2, Table S1). The nearly ubiquitous presence of β-amyrin synthases and lupeol synthases that form one large group indicates that some triterpenes of the oleanane or lupane series may have a general physiological significance beyond the species-specific well-known accumulation of triterpene derivatives, such as saponins in *Gypsophila trichotoma* [12]. This has been described recently in a series of studies describing the effect of triterpene lipids on the structure of cuticular lipopholic barriers in *Arabidopsis thaliana* [13], or the role of these triterpenes as epicuticular crystals in the mediation of plant-insect interactions in a species-specific manner [14]. In addition, functional approaches in planta have assigned specific roles for some of these triterpene synthases in the production of β-amyrin as a precursor of phytoanticipins like the avenacin saponins in oat [15]. CAS1 proteins cluster into distinct entities indicating their apparenence to land plants, algae, or protists (Fig. 2). A distinct group of proteins is formed by OSCs that have been characterized as LAS1 from plants owing to the complementation of the yeast lanoster synthase deficient *erg7* mutant.
The biochemical characterization of a wealth of triterpene synthases (or OSCs) isolated from many different plant species revealed in addition to CAS1 and OSCs that produce triterpenoids of diverse structure, the general presence of LAS1 in the plant OSCs repertoire [17,18]. The increasing number of genome sequences available in the databases is constantly reinforcing this fact. The contribution of a lanosterol pathway to the production of 24-alkyl-Δ5-sterols was shown to be marginal even upon strong expression of LAS1 in transgenic *Arabidopsis thaliana* [19]. Moreover, the contribution of lanosterol as a precursor of natural products remains unclear until now [20]. In fact, lanosterol has been only detected in large amounts in the latex of *Euphorbia* species [21]. The restriction of this sterol precursor to the complex *Euphorbia* genus has not yet received any functional explanation. It is nonetheless worth noting that 2-tritio lanosterol and 2-tritio cycloartenol when fed to *Sorghum bicolor* leaves were converted to cholesterol and sitosterol even though cycloartenol was a more effective sterol precursor than lanosterol [22]. Thus, the presence of a lanosterol metabolism in plants, possibly related to phytosterol biosynthesis, deserves further investigations.

The essential role of CAS1 in sterol biosynthesis and plant development was demonstrated by a series of *Arabidopsis thaliana* hypomorphic or conditional alleles [23]. Such plants developed an albino phenotype in tissues that undergo active cell division and elongation such as leaf margins and apices. The negative effect upon plastid development was strictly coincidental with an accumulation of 2,3-oxidosqualene. The CAS1-dependent sterol biosynthetic segment consists of a succession of 4 enzymatic steps leading from cycloartenol to cycloeucalenol (Fig. S1). This upstream segment is connected with the downstream segment leading to 24-alkyl-Δ5-sterols by the enzyme CPI (cyclopropyl isomerase, EC 5.5.1.9), being able to isomerize cycloeucalenol into the tetracyclic 4x-methyl sterol, obtusifoliol [24,25]. Obtusifoliol is further converted into phytosterols in a way that is reminiscent of the conversion of lanosterol to ergosterol in metazoans [8,9]. This CAS1 segment is highly conserved in photosynthetic organisms and beyond, in protists. The strong phenotype of an *Arabidopsis thaliana* cpsi-1 mutant indicates that a normal life span cannot be attained with the CAS1 biosynthetic segment only [26], although growth of cells [27] and to a limited extent of plants with 9β,19-cyclopropylsterols [28] have been reported.

The co-existence of functional CAS1 and LAS1 in plants requires further studies. So far, in *planta* characterization of CAS1 and LAS1 was restricted to *Arabidopsis thaliana*. In order to further document dual cycloartenol and lanosterol pathways in higher plants we have isolated a CAS1 from *Nicotiana tabacum* and studied its function in yeast and in *Nicotiana benthamiana*. We used virus induced gene silencing (VIGS) based on gene specific sequences derived from the solgenomics initiative (http://solgenomics.net/) to challenge the respective roles of CAS1 and LAS1 in *Nicotiana benthamiana*. In this report, we show a strict dependence on CAS1 of tobacco sterol biosynthesis.

### Results and Discussion

**Genes and cDNAs encoding 2,3-oxidosqualene cyclases (OSCs) in *Nicotiana tabacum* and *Nicotiana benthamiana***

We surveyed the genome databases to identify *Nicotiana* orthologs of CAS1 and LAS1. We then cloned a *Nicotiana tabacum* CAS1 cDNA by recursive PCR using degenerated primers over conserved sequences. Primers used for that cloning procedure are given Table S2. The full length cDNA encoded a 2856 bp open reading frame displaying 78% identity with AtCAS1 and consequently was named *NicASI* (GenBank accession KM452913). We next identified in the genome of *Nicotiana benthamiana* (http://solgenomics.net/) two genes whose products had 70% and 58% identity with AtCAS1 and AtLAS1, respectively. Relevant sequences identified in *Nicotiana* and in other Solanaceae are given in Table S3. It is worth noting that probing the genome of *Nicotiana benthamiana* with the functionally identified tomato triterpene synthases [29] resulted in the identification of a predicted protein (NbS00041716g0004.1) that exhibited 85% identity with the tomato β-amyrin synthase and 77% with the *Arabidopsis thaliana* β-amyrin synthase (Table S3). The presence of a putative β-amyrin synthase gene in the *Nicotiana benthamiana* genome certainly requires further investigations to determine its physiological relevance since β-amyrin has not been detected in *Nicotiana benthamiana* leaves in normal physiological conditions [30]. The simple structure of the OSC gene family in *Nicotiana benthamiana* makes this plant an excellent model to further investigate functional aspects related to CAS1 and LAS1 genes.

We cloned by RT-PCR a cDNA fragment from *N.benthamiana* leaf RNA, that corresponded to an expressed *NbLAS1* (Table S3), confirmed by qRT-PCR analysis. The alignment of amino acid sequences of CAS1 and LAS1 from *Nicotiana tabacum*, *N.benthamiana*, *Solanum lycopersicon* and *Capsicum annuum* (Fig. 3) shows features that support the identification of CAS1 and LAS1 sequences in these solanaceae. In fact, these two enzymes have been studied extensively. Molecular evolution and site-directed mutagenesis experiments revealed important aminoacid residues and conserved motifs that govern the cyclization of OSC into cycloartenol or into lanosterol. All CAS1 enzymes have a strict requirement for His477 and I481 (*Arabidopsis* numbering) in the vicinity of the conserved DCTAE motif implicated in substrate protonation, whereas all LAS1 proteins have a strict requirement for a V401 (Fig. 3) [31,32]. A thorough analysis of the cyclization reactions, which required the heterologous expression of the plant CAS1 or LAS1 enzymes in the yeast *erg7* (lanosterol-deficient) mutant led to a current understanding of the catalytic differences in both reactions [33].

**Functional characterization of CAS1 and LAS1 in *Nicotiana benthamiana***

To ascertain the function of CAS1 and LAS1 in *N. benthamiana*, we silenced the corresponding genes in expanding leaves of young plants using VIGS. For this we cloned gene-
specific cDNA fragments of *NtCAS1* and *NbLAS1* into a PVX-based vector. Transcripts generated from *PVX*, *PVX::CAS1*, and *PVX::LAS1* constructs were then inoculated into leaves by wounding. Visual inspection of inoculated plants showed very clearly the appearance of a strong phenotype for *PVX::CAS1* plants. The first effects on growth were a general reduction of plant stature and a leaf bleaching that was prominent on veins (Fig. 4A). In the most severe cases, plant leaves and other tissues that had developed after the inoculation of the *PVX* transcripts to the first and second leaf pair displayed a strong wilting and necrotic zones (Fig. 4B). This impaired any further growth and development. Conversely, *PVX::LAS1* plants had a lifespan and morphological aspect identical to *PVX*- or mock-inoculated plants (Fig. S1). Gene expression analysis carried out by RT-qPCR indicated a massive accumulation of the viral transcripts *PVX::CAS1* and a reduction of the endogenous *CAS1* messenger RNA in *PVX::CAS1* plants (Fig. 4C). Closely similar results were obtained for the expression of *LAS1* that was reduced in *PVX::LAS1* plants concurrently to a huge load of viral *PVX::LAS1* transcripts (Fig. 4D). Next, measurements of squa-}

**Figure 2. Phylogenetic tree illustrating the distribution of 2,3-oxidosqualene cyclases in plants, algae, and protists.** The distance between each sequence was calculated using the program CLUSTAL W. The phylogenetic tree was drawn using PhyloTol (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html). Scale represents 0.1 amino acid substitutions per site. Protein clusters are enhanced by colors as follows: red and orange, β-

amine and promiscuous β-

amine synthases; dark red, lupeol synthases; blue, plant lanosterol synthases; green, plant cycloartenol synthases; brown, cycloartenol synthases from algae or a plantecycte, and yellow, cycloartenol synthases from protists. Abbreviations and accession numbers are given in Table S1. All proteins displayed in the phylogenetic tree have either been functionally characterized or genome annotated (except CAS1 from *Salpingoeca sp*, *Allium macrosternon*, *Avena strigosa*, *Avena ventricosa* and *Luffa cylindrica*, which have been annotated based on sequence homology and/or expression evidence).

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in PVX::CAS1 leaf tissues most probably allowed this \( \beta \)-amyrin synthase to access to a pool of that substrate. It is worth noting that such a strong accumulation of 2,3-oxidosqualene precludes reliable enzymatic analyses using subcellular fractions.

**NtCAS1 defines a functional protist/plant sterol pathway in yeast**

The functional identification of *NtCAS1* as encoding a cycloartenol synthase was further performed by its expression in the yeast mutant *erg7* that lacks an endogenous lanosterol synthase [34]. Upon galactose-induced expression of *NtCAS1*, yeast cells grown in the presence of exogenous ergosterol displayed a sterol profile (Fig. 5A and B) that included cycloartenol (peak 3) and four other sterols (peaks 4 to 7) in addition to ergosterol (peak 2). The major sterol was 24-methylene pollinastanol (peak 7) that accounted for 53% of the total and represented the most probable pathway end-product generated by the yeast steroidogenic machinery, apparently being versatile enough to accept cycloartenol as a substrate. The most probable sterol pathway of *erg7::NtCAS1* is shown in Fig. 5C. Cycloartenol is demethylated at C-4 by the demethylation complex [35] to produce 31-mono-cycloartenol, which is further taken by the sterol-C24-methyl-transferase (ERG6) to produce cycloeucalenol (peak 4) or by the C-4 demethylation complex to remove the second methyl group at C-4 to yield 24-methylene pollinastanol (peak 7). The favored grid of possible interconversions presented in Fig. 5C is in agreement with previous work done with exogenously cycloartenol-fed yeasts [36] or more recently with the expression of a rice CAS1 in yeast, this to a certain extent [37]. The absence of 24-methylene cycloartenol (the direct product of a possible action of ERG6 on cycloartenol) may indicate that the recruitment of the yeast C4-demethylation enzyme complex (ERG25, ERG26, ERG27 and ERG28) occurs prior to the side chain alkylation, or that 24-methylene cycloartenol has a rapid turnover [38]. As it is the case for all enzymatic conversions implied in this pathway, the methylation at C24 was partial, which may explain the identification of 24-dehydropollinastanol as well (peak 6). Interestingly, no sterols bearing a reduced side chain at \( \Delta^{24} \) were detected, indicating a probable exclusion of ERG4, the yeast \( \Delta^{24} \)-reductase, from the cellular compartment involved in the yeast 9\( \beta \),19-cyclopropylsterol biosynthesis. In this respect, it was demonstrated over the last years that the yeast sterol biosynthetic segments are seemingly localized in distinct compartments, of which the lipid droplets are major players in hosting ERG6, ERG7 and the C-4 demethylation complex [39]. Thus, the sterol biosynthetic features of *erg7::NtCAS1* were identical to the 9\( \beta \),19-cyclopropylsterol biosynthetic segment of protists, algae, or
plants. Cell cultures of these latter biological models were shown to grow, albeit partially, only with that biosynthetic segment, as seen after chemical inhibition of the cyclopropyl isomerase that links the 9β,19-cyclopropylsterol biosynthetic segment to the downstream tetracyclic segment of the sterol pathway [27]. Indeed, those eukaryotic cells could live with 4,4-demethylated cycloartenol derivatives such as 24-methylene- and 24-methyl-pollinastanol. For these reasons, the growth of erg7::NtCAS1 was examined on a minimal selective medium in the presence or absence of ergosterol (Fig. 6). Growth assessment performed as spotting assays was made after five days. In the presence of ergosterol, erg7 or erg7::NtCAS1 displayed a closely similar growth profile along a range of dilutions, whereas in the absence of ergosterol, only erg7::NtCAS1 was able to grow significantly when compared to erg7. The growth was however slowed down as shown by the significantly reduced spots. This reinforces previous studies showing that cycloartenol cannot support growth of yeast unless it has been metabolized to C-4 desmethyl sterols such as 24,25-dehydropollinastanol [40]. Such result was in agreement as well with previous observations [27] about 9β,19-cyclopropylsterols being good surrogates to tetracyclic Δ5-sterols (cholesterol, ergosterol, sitosterol) in a way that they do not prevent a yeast cell or a plant cell to divide. Nonetheless, cellular and morphogenetic inhibitions of multicellular organisms depleted in 24-alkyl-Δ5-sterols were severe [23,26].

Conclusions

VIGS is a powerful tool to challenge the biological function of genes [41]. The most popular model plant that is currently the VIGS workhorse for biochemical studies is Nicotiana benthamiana (http://bti.cornell.edu/nicotiana-benthamiana/). Specific silencing of genes [42], analysis of plant-pathogen interactions mediated by sphingolipid biosynthesis [43], and genetic screens [44] have been described. We show here using VIGS that the tobacco cycloartenol synthase gene CAS1 encodes the only OSC essential to sterol biosynthesis. Its decreased expression results in impaired development, bleaching of leaf veins, severe leaf wilting, vegetative and reproductive development arrest. This is in full agreement with previous studies that demonstrated the vital role of CAS1 in Arabidopsis thaliana owing to a genetic mosaic analysis [23]. Furthermore, the morphogenetic inhibition developed by PVX::CAS1 Nicotiana benthamiana leaves is closely similar to the severe stunting of PVX::CYP51 plants, due to a lack of 24-alkyl-Δ5-sterols upon inhibition of NtCYP51 that encodes obtusifoliol-14-demethylase [42]. However, the strong albini

Materials and Methods

RT-PCR cloning of cDNAs

Total RNAs from 7-day-old TBY-2 cells or from Nicotiana benthamiana young leaves were extracted and purified with Trizol reagent (Invitrogen) or with the NucleoSpin RNA Plant kit (Macherey-Nagel), following the manufacturer’s recommendations. NtCAS1 full length cDNA obtained by recursive PCR
cloning (primers in Table S2) was subcloned in pBluescript vectors and sequenced according to standard molecular biology methods.

Virus-Induced Gene Silencing

Potato Virus X vectors were engineered to achieve the silencing of *Nicotiana benthamiana* *CAS1* and *LAS1* genes. A 1 kb fragment of the *NicAS1* cDNA (nucleotides 958 to 2023 of the CDS, GenBank accession KM452914) was obtained by EcoRV digestion of the pBluescript::*NicAS1* cloning vector. A 0.45 kb fragment designed from the first 470 nucleotides of the *NbLAS1* cDNA retrieved from the solgenomics database (http://solgenomics.net/, NbS00053226g00006) was isolated by RT-PCR using primers 5′-GGGAAACGAAACCTTTYGGG-3′ (forward) and 5′-GGTAACACGATGTATCA-3′ (reverse) formatted directionally into ClaI and SalI, respectively (GenBank accession KM452913). Purification and subcloning of cDNA fragments was done according to standard cloning procedures. The 1 kb *CAS1* fragment was inserted into the EcoRV site of the pP2C2S vector [45], the 0.45 kb *LAS1* fragment was subcloned into the ClaI (5′ end) and SalI (3′ end) sites of the same vector. To generate high amounts of PVX-derived transcripts, pP2C2S constructs (10 µg) were linearized via SpeI restriction and used as template for an in vitro transcription reaction that was done with the Ribomax large scale RNA production system T7 kit (Promega). Transcription was followed by UV spectrophotometry.

**Figure 6. Yeast spotting assay with ERG7 deficient gil77 or gil77::CAS1 strains.** Growth of gil77 yeast strain transformed with the pYeDP60 vector or pYeDP60-NtCAS1 was tested on SGal medium supplemented or not with 20 µg/ml of ergosterol. After 5 days at 30°C, plates supplemented with ergosterol showed fully-grown colonies, whereas inductive conditions (SGal without ergosterol) allowed only limited growth of yeast expressing NtCAS, indicating however that NtCAS1 expression partially overcomes ERG7 deficiency. doi:10.1371/journal.pone.0109156.g006

Sterol and triterpene extraction and analysis

Freeze-dried yeast or plant samples (50 to 100 mg) were saponified for one hour in a 6% potassium hydroxide methanolic solution at 80°C. The unsaponifiable fraction was extracted with hexane then filtrated through a 0.45 µm PTFE membrane (Pall Life Sciences). The dried extract was acetylated in toluene with a mixture of pyridine/acetic anhydride. After evaporation of the reagents, a 5973N instrument (Agilent) equipped with a DB5-MS column and coupled to a 6853 mass analyser (Agilent). The temperature program of ovens included a steep ramp at 30°C/min from 60°C to 220°C then a 2°C/min increase from 220°C to 300°C. Sterols were unequivocally identified by coincidental retention time and identical EI-MS spectra at 70 eV like reference compounds as described [47].
Supporting Information

Figure S1  Plant sterol biosynthetic pathway. The biosynthetic segment framed in red is the so-called 9β,19-cyclopropylsterol segment that requires four enzymes from cycloartenol : SMT1, cycloartenol-C24-methyltransferase [S1]; SMO1, sterol methyl oxidase [S2]; 3βHSD/D, 3β-hydroxysteroid/C4-decarboxylase [S3]; SR, sterone reductase [S4]. The CP11, cycloprenyl isomerase, links the 9β,19-cyclopropylsterol biosynthetic segment to the metazoan-type sterol pathway that leads to 24-alkyl-Δ5 sterols (campesterol and sitosterol). Uncomplete methylation of the sterol side chain by SMT1 leads to the cholesterol pathway (not shown here for clarity of the figure).

Figure S2  Morphological phenotype of (A) PVX::LAS1 and (B) PVX::CAS1 plants 4 weeks after inoculation. The picture is representative of 4 independent experiments that included all 3 plants inoculated with each type of viral transcripts. The distribution of sterols in control PVX, PVX::CAS1 or PVX::LAS1 is shown in (C).

Figure S3  GC-MS analysis of PVX, PVX::CAS1, and PVX::CAS1 unsaponifiable acetylated acetates showing the presence of β-aminoyl in PVX::CAS1. TIC between 22 min and 39 min are shown for (A), PVX; (B), PVX::LAS1, (C), PVX::CAS1. Compounds (as acetate derivatives) are : 1, cholesterol; 2, campesterol (the shouldering peak is 24-methylene sterols (campesterol and sitosterol). Uncomplete methylation of the sterol side chain by SMT1 leads to the cholesterol pathway (not shown here for clarity of the figure).

References


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Author Contributions

Conceived and designed the experiments: EGP TJB HS. Performed the experiments: EGP AB. Analyzed the data: EGP HS. Wrote the paper: EGP HS.