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ORIGINAL ARTICLE

Inhibition of Cycloartenol Synthase (CAS) Function in Tobacco BY-2 Cells

Elisabet Gas-Pascual[2] · Biljana Simonovik[1] · Hubert Schaller[1] · Thomas J. Bach[1]

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Abstract Tobacco BY-2 cell suspensions are our preferred model for studying isoprenoid biosynthesis pathways, due to their easy genetic transformation and the efficient absorption of metabolic precursors, intermediates, and/or inhibitors. Using this model system, we have analyzed the effects of chemical and genetic blockage of cycloartenol synthase (CAS, EC 5.4.99.8), an oxidosqualene cyclase that catalyzes the first committed step in the sterol pathway of plants. BY-2 cells were treated with RO 48-8071, a potent inhibitor of oxidosqualene cyclization. Short-term treatments (24 h) resulted in accumulation of oxidosqualene with no changes in the final sterol products. Interestingly, long-term treatments (6 days) induced down-regulation in gene expression not only of *CAS* but also of the *SMT2* gene coding sterol methyltransferase 2 (EC 2.1.1.41). This explains some of the increase in 24-methyl sterols at the expense of the 24-ethyl sterols stigmasterol and sitosterol. In our alternative strategy, *CAS* gene expression was partially blocked by using an inducible artificial microRNA. The limited effectiveness of this approach might be explained by some dependence of the machinery for RNAi formation on an operating MVA/sterol pathway. For comparison we checked the effect of RO 48-8071 on a green cell suspension of Arabidopsis and on seedlings, containing a small spectrum of triterpenes besides phytosterols. Triterpenes remained essentially unaffected, but phytosterol accumulation was clearly diminished.

Keywords Tobacco BY-2 cells · Cycloartenol synthase · RO 48-8071 · Sterol analysis · amiRNA · Gene expression

Abbreviations

CAS	Cycloartenol synthase
NtCAS	Nicotiana tabacum CAS
RO 48-8071	(4-Bromophenyl)[2-fluoro-4-[[6-(methyl-2- propenylamino)hexyl]oxy] phenyl]-methanone
SMT	Sterol methyltransferase
amiRNA	Artificial micro interfering RNA
RNAi	Interfering RNA
microRNA	Small interfering RNA
MVA	Mevalonic acid
BY-2 cells	(Tobacco) Bright Yellow-2 cells
LAS	Lanosterol synthase
MEP	Methyl erythritol phosphate
PFTE	Polytetrafluoroethylene
GC-FID	Gas chromatography with flame ionization detector

Introduction

As major end products of the cytoplasmic mevalonic acid (MVA) pathway, phytosterols play a vital role in the maintenance of membrane structures [1] under various

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✉ Thomas J. Bach
bach@unistra.fr

[1] Institut de Biologie Moléculaire des Plantes, CNRS UPR 2357, Université de Strasbourg, 28, rue Goethe, 67083 Strasbourg, France

[2] Present Address: Department of Horticulture and Crop Science, The Ohio State University, Wooster, OH 44691, USA

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environmental regimes. They are functional in the growth and development of plants [2, 3] as precursors of steroid hormones [2, 4]. Phytosterols also are involved in plant-pathogen interactions [5] and in the synthesis of cellulose [6, 7]. Their role in the polar transport of auxin has been described [8], as is their importance in epigenetic regulation by the microRNA system [9]. Their enrichment in Triton X-100 insoluble micro-domains (lipid rafts) localized to the plasma membrane has been demonstrated [10]. Such rafts might present a suitable microenvironment for specific enzymes and receptors at or in the cell plasma membrane.

The biosynthesis pathway leading to phytosterols in higher plants has been elucidated within the last two decades [1, 11, 12], but there are still gaps in our knowledge as to regulation and accumulation. The first committed step in phytosterol biosynthesis is catalyzed by cycloartenol synthase (CAS, EC 5.4.99.8), with oxidosqualene as substrate. Even though the presence of a lanosterol synthase (LAS, EC 5.4.99.7) in plants has been confirmed [13, 14], an enzyme that was thought to exist only in animals and fungi, its contribution to the total synthesis of phytosterols remains negligible in most of the plants investigated [15]. An exception to this rule is the latex of *Euphorbia lathyris*, which accumulates considerable quantities of lanosterol and a number of triterpenes [16]. In the green alga *Chlamydomonas reinhardtii* a CAS does exist, but the final sterol that accumulates is ergosterol, like in fungi, where the pathway proceeds through lanosterol as an intermediate [17]. In this case, due to the absence of the mevalonic acid (MVA) pathway in green algae, ergosterol synthesis must rely on the prokaryotic methyl erythritol phosphate (MEP) pathway for the delivery of isoprene units [18, 19].

In plants, CAS is membrane-localized along with squalene synthase, squalene epoxidase, and downstream enzymes in the phytosterol pathway. Also, the membrane localized is an "early" enzyme in the MVA pathway, HMG-CoA reductase, which is considered a coarse-regulator of the endomembrane-associated phytosterol pathway [20]. However, fine-tuning of later steps also is likely involved in regulation [21]. It has long been hypothesized that such enzymes are not randomly distributed throughout the endomembrane system, but rather form metabolic units ("metabolons") [22].

Recently an allelic series of *cas1* mutations of Arabidopsis was characterized [23]. Plants carrying the weak mutant allele *cas1-1* were viable but developed albino inflorescence shoots, due to photooxidation of plastids, paralleled by an accumulation of oxidosqualene [23]. Two strong mutations, *cas1-2* and *cas1-3*, were not transmissible through the male gametes, suggesting a role for CAS1 in male gametophyte function [23]. Also, *cas1* mutations affected plastid biogenesis [23].

A further approach to study the functional importance of a particular enzyme reaction is its inhibition by specific inhibitors [24–30], or by down-regulation of gene expression [30–32]. In this contribution we report on the inhibition by RO 48-8071 [35] and gene down-regulation strategies for the functional study of tobacco CAS in relation to growth and phytosterol accumulation. We use the well-established system of dark-grown, chlorophyll-free tobacco BY-2 suspension cells, which grow rapidly with a duplication time (complete cell cycle) of about 14 h [33]. These cells have been experimentally evaluated for their rapid uptake of nutrients like glucose and sucrose, but also of inhibitors and specific precursors in isoprenoid biosynthesis [34].

Materials and Methods

Gene Isolation and amiRNA Plasmid Construction

Using the available information on tobacco EST sequences, as well as sequence information of *CAS* genes from other eudicot species (*N. tabacum* putative *CAS* EST, AM842317, AM822688 and AM831068*; CAS1 Betula platyphylla*, AB055509*; Taraxacum officinale,* AB025346*; Luffa cylindrica,* AB033334*; Ricinus communis,* DQ268870*; Panax ginseng,* AB009029*; Arabidopsis thaliana,* U02555*; Centella asiatica,* AY520819; *Pisum sativum*, D89619; *Glycyrrhiza glabra*, AB025968), we designed a set of degenerate primers (NtCAS for 5′-AGGAAGAYYTDTACTAYCCWCAYC-3′ and NtCAS rev 5′-CGRTATTCTCCHARNGCCCA-3′). Using these primers on 7-day-old BY-2 cells cDNA, we amplified a 1300 bp fragment with homology to the 3′ region of the already described *CAS* genes. This partial sequence was submitted to WMD2 (http://wmd2.weigelworld.org/cgi-bin/mirnatools.pl) for prediction of artificial microRNA (amiRNA). We selected three amiRNA sequences as a silencing strategy for *CAS* gene in tobacco, amplified them by recursive PCR as described [36], and cloned them into a pBSK vector (see Table 1 for oligo sequences). These amiRNA sequences (Table 1) were then subcloned via SpeI/XhoI into the pER8 estradiol-inducible vector (kindly provided by Prof. Nam-Hai Chua, Rockefeller University, New York) for its inducible expression into BY-2 cells [37].

Plant Materials

BY-2 Cell Culture, Transformation, and Estradiol Induction

The tobacco (*Nicotiana tabacum* cv BY-2) suspension cell culture was originally made available by Prof. Toshiyuki Nagata (Tokyo University, Japan). Cell suspensions

Table 1 Oligos used for amiRNA cloning

Name	Sequence	bp
CAS 1.I	gaTATCATCTAACACCTGTGCATtctctcttttgtattcc	40
CAS 1.II	gaATGCACAGGTGTTAGATGATAtcaaagagaa tcaatga	40
CAS 1.III	gaATACACAGGTGTTTGATGATTtcacaggtcgtg atatg	40
CAS 1.IV	gaAATCATCAAACACCTGTGTATtctacatatatattcct	40
CAS 2.I	gaTTATGTTGCAAAGCGACCACGtctctcttttgtattcc	40
CAS 2.II	gaCGTGGTCGCTTTGCAACATAAtcaaagagaa tcaatga	40
CAS 2.III	gaCGCGGTCGCTTTGGAACATATtcacaggtcgtg atatg	40
CAS 2.IV	gaATATGTTCCAAAGCGACCGCGtctacatatatattcct	40
CAS 4.I	gaTCATAATGAATGTGGTCCGTCtctctcttttgtattcc	40
CAS 4.II	gaGACGGACCACATTCATTATGAtcaaagagaa tcaatga	40
CAS 4.III	gaGAAGGACCACATTGATTATGTtcacaggtcgtg atatg	40
CAS 4.IV	gaACATAATCAATGTGGTCCTTCtctacatatatattcct	40

were maintained by weekly dilution (1:40) of cells into fresh medium as described by Nagata et al. [34] and cultured in the dark at 26 °C with shaking at 140 rpm. The pER8-amiRNA constructs were introduced into the *Agrobacterium tumefaciens* LBA4404 hyper-virulent strain and transformed into BY-2 cell suspensions via co-culture as previously described [38, 39]. Clonal cell cultures were established from isolated calli on Murashige and Skoog (MS) medium modified for BY-2 as previously described [38, 39], supplemented with hygromycin B (30 µg/mL), and screened by PCR to confirm t-DNA insertion with oligos pER for 5′-GCTCGACTCTAGGATCTTCG-3′ and pER rev 5′-GTAGGATTCTGGTGTGTGG-3′.

To induce miRNA expression, 3 mL of BY-2 cells at different phases of the cell growth curve (1–5 days after subculture) was dispensed into 6-well culture cluster plates (Corning, obtained via Sigma-Aldrich) and treated with 5 µM estradiol (Sigma-Aldrich). Treated cells were then placed on a rotary shaker at 150 rpm and kept in darkness at 26 °C for 15 h (standard condition, empirically determined), then collected by centrifugation, and deep frozen in liquid N_2. Frozen pellets were used for RNA extraction with Trizol reagent (Invitrogen) following the manufacturer's recommendations.

BY-2 Cell Treatment with RO 48-8071 and Estradiol

For experiments with RO 48-8071, the working concentration in BY-2 cells was empirically determined by analyzing the growth curve at 7 days after treatment with 1, 2, 5, or 10 µg/mL of RO 48-8071 (5 µg/mL corresponds to 11.15 µM). Cells were subcultured, treated with the indicated amounts of inhibitor, and harvested by vacuum filtration 1–7 days after treatment. The packed cell volume was calculated and 2 µg/mL was chosen for subsequent experiments as the inhibitor concentration only marginally affected cell growth.

Selected *NtCAS1* silenced lines 1.8 and 4.8, as well as the pER8.1 empty vector line, and were grown in the dark at 26 °C, at 150 rpm shaker speed, for 3 days after subculture. Transgene expression in BY-2 cells was induced with 5 µM estradiol and induced and non-induced cell cultures were incubated in the dark at 26 °C and 150 rpm. Induced and non-induced BY-2 cells were collected by vacuum filtration and stored at −80 °C. Unless indicated otherwise, cells were harvested after 1 or 6 days following RO 48-8071 treatment and/or estradiol induction.

Analysis of Chemically Treated/Silenced BY-2 Cells

Freeze-dried BY-2 cells (100–800 mg) were ground in liquid N_2 and saponified for 1 h in a 6 % potassium hydroxide methanolic solution at 80 °C. The unsaponifiable fraction was extracted with hexane, and then filtered through a 0.45 µm PFTE membrane (Pall Life Sciences). The dried extract was acetylated in toluene with a mixture of pyridine/acetic anhydride (5:2.5:1, v/v/v) for 2 h at 70 °C. After evaporation of the reagents, a known amount of betuline diacetate (Apin Chemicals Ltd, Abington, UK) was added as an internal standard for GC-based quantification. GC-FID used for quantification was performed with a 3400CX gas chromatograph (Varian) equipped with a DB-5 column (30 m wall-coated open tubular, 0.32 mm i.d., 250 µm film thickness, H_2 flow rate 2 mL/min). The temperature of the injector and detector was 250 and 300 °C, respectively. Compounds were identified by GC–MS using a 6890 instrument (Agilent) equipped with a DB5-MS column and coupled to a 5973 mass analyzer (Agilent). The temperature program of ovens included a steep ramp at 30 °C/min from 60 to 220 °C, then a 2 °C/min increase from 220 to 300 °C. Sterols were unequivocally identified by coincidental retention time and matching EI-MS spectra at 70 eV as were the reference compounds that were previously isolated, characterized, and stored in our laboratory [40].

Arabidopsis Thaliana MMD2 Cell Culture and Chemical Treatment

The *Arabidopsis thaliana* MM2D cell suspension was a gift from Dr. Marie-Claire Criqui (Institut de Biologie Moléculaire des Plantes, Strasbourg). This cell suspension was originally established by Menges and Murray [41]. Essentially, MM2D cells were subcultured (1/20) in 80 mL fresh medium (MS basal medium, 3 % sucrose, 0.5 mg/L

NAA, 0.05 mg/L kinetin). Cells were incubated under a 16-h light/8-h dark photoperiodic regime, with gentle shaking at 125 rpm, at 24 °C. One day after subculture, cells were treated with either acetone or 5 µg/mL RO 48-8071, dissolved in equal quantities of acetone. After 3 days of treatment, cells were harvested by vacuum filtration, rinsed with PBS, deep-frozen in liquid N_2 and lyophilized. Freeze-dried cells (50–200 mg) were ground and the sterol composition was then analyzed as described for tobacco BY-2 cells.

Arabidopsis Thaliana Growth Conditions and Chemical Treatment

Arabidopsis thaliana (Col-0) seeds were surface sterilized and sown on MS medium plates supplemented with 1 % sucrose. Plates were supplemented with either 5 µg/mL RO 48-8071 dissolved in acetone or with equal amounts of acetone. Seedlings were grown under LD conditions (16-h light/8-h dark), at 23 °C during the day and 21 °C during the night. After 3 weeks, seedlings were harvested, freeze-dried, and ground. Triterpenoid analysis was performed essentially as described [23].

Molecular Characterization of the Inhibited/Silenced BY-2 Cell Lines

Gene expression was analyzed by quantitative reverse transcriptase-mediated Real Time PCR (qRT-PCR). RNA was extracted from dried BY-2 cells with the Nucleospin RNA Plant kit (Machenary-Nagel) and cDNAs were synthesized from 1 µg total RNA by using a SuperScript III Retro transcription kit (Invitrogen). For *NtCAS1* and *NtSMT2* and *HMGR,* gene expression was analyzed by qRT-PCR, using SYBR green (MESA GREEN + fluorescein, Eurogentec) either in an iCycler thermal cycler (Biorad) or a Light Cycler 480 (Roche). *NtEF1* and *NtACT* were used as control genes. Primers used in the qPCR analysis were designed either with Primer3 public software (http://frodo.wi.mit.edu/primer3/) or with the Light Cycler probe design tool (Roche), and their sequences are shown in Table 2. Experiments were repeated at least three times. RNA quality was monitored by UV spectrometry.

Table 2 Oligos used for qPCR analysis

Name	Sequence (5′–3′)	Tm (°C)
ACT qPCR for	TGATAACGGAACAGGAATGG	62
ACT qPCR rev	TCGAGGTCGACCAACAATAC	63
CAS1 qPCR for	CCTTCCAATTCATCCTGGAA	64
CAS1 qPCR rev	GCCAACGAACCTTTTACCAT	63
EF1 qPCR for	TACCACCCCCAAGTATTCCA	64
EF1 qPCR rev	TGTTGTCACCTTCCAAACCA	64
HMGR1 qPCR for	GTTTCCTCTCCCAAGCATC	62
HMGR1 qPCR rev	GGCGTCGAATTACGGATCTT	65
HMGR2 qPCR for	CAGGACAACTCCCTTCTCAT	61
HMGR1 qPCR rev	GGAGTGGAGTTCCTGATTTT	60
SMT2.2 qPCR for	CAGTGTGAAGTTGTTTGTGGTA	63
SMT2.2 qPCR rev	ATGTTGCTTCAATGG AATAAACTC	62

Results

Establishment of Suitable Experimental Conditions

In our study we used RO 48-8071, which efficiently blocks the plant-specific CAS (see Fig. 1 for the inhibitor's structure and the site of inhibition in the phytosterol pathway) to elucidate the functional importance of cycloartenol formation in the simplified plant system of tobacco BY-2 cells. We first determined the best working concentrations in BY-2 cell suspensions (Fig. 2). After starting a new culture from 7-day-old cells in stationary phase, exponential growth started between day 3 and 4, reaching steady state at day 8 by the latest. At 2 µg/mL (~4.46 µM), growth inhibition became apparent after 24 h (Fig. 2a), entry into log phase was delayed, but cell growth recovered to about 80 % after 7 days (Fig. 2a). At 5 µg/mL, and higher concentrations below 20 µg/mL, BY-2 cells remained inhibited and only recovered growth to a limited extent. At 20 µg/mL, cell growth was completely arrested (Fig. 2a) with no recovery. For comparison, we treated *N. benthamiana* seedlings cultured on agar medium supplemented either with acetone (control, Fig. 2b) or 2, 5, and 10 µg/mL of inhibitor dissolved in acetone (Fig. 2b–e). Partial inhibition of growth and development was apparent at the lowest concentration.

Sterol Analysis

Phytosterols and their precursors were analyzed in control and treated BY-2 cells after 24 h (Fig. 3a) and after 6 days (Fig. 3b) of exposure. In the initial phase of resuming growth following transfer of 7-day-old cells into a new medium, phytosterol content was comparably low and remained essentially unaffected by the inhibitor at 24 h, although the appearance of squalene and oxidosqualene clearly indicates that the CAS was inhibited in vivo. This spectrum changed after 6 days of exposure with the appearance of 24-methylene cholesterol, and a 40 % decline in combined (Δ^5) ($24\alpha = 24R$) campesterol and its 24β-methyl epimer, (Δ^5) ($24\beta = 24S$) 22-dihydrobrassicasterol [which cannot be resolved by GLC or HPLC (cf. [42] and references therein)]. However, the inhibitory effect on

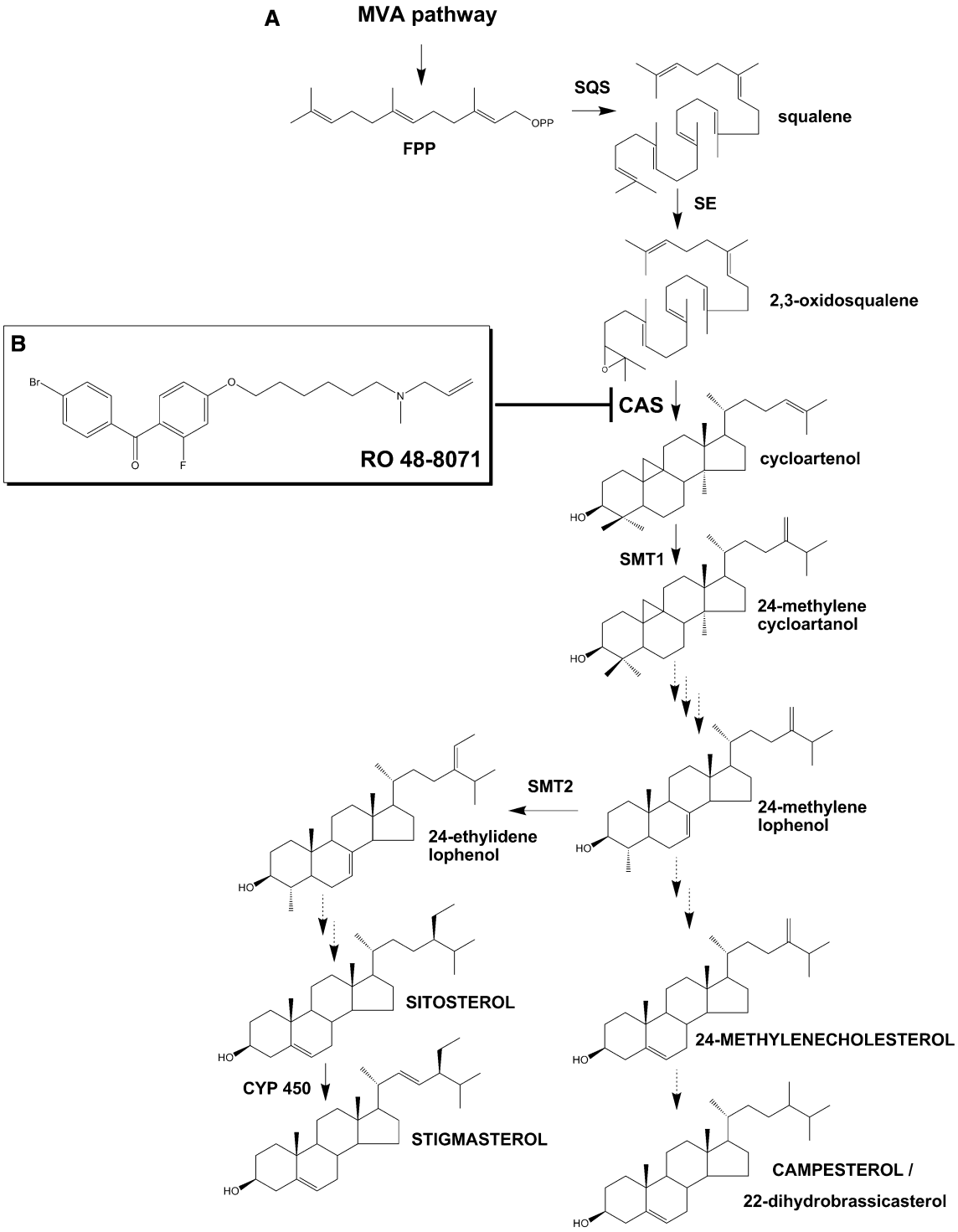

Fig. 1 **a** Sterol biosynthesis in plants. The building blocks for plant sterol biosynthesis are provided by the mevalonate (MVA) pathway. Two molecules of farnesyl diphosphate (FPP) are condensed to yield squalene, the first specific intermediate of the pathway. The first three committed steps of sterol biosynthesis are highlighted: SQS (squalene synthase), SE (squalene epoxidase) and CAS (cycloartenol synthase). The two methylation steps by the two sterol methyltransferases (SMT1 and SMT2) are also indicated. The site of RO 48-8071 inhibition is shown. **b** Structure of the inhibitor

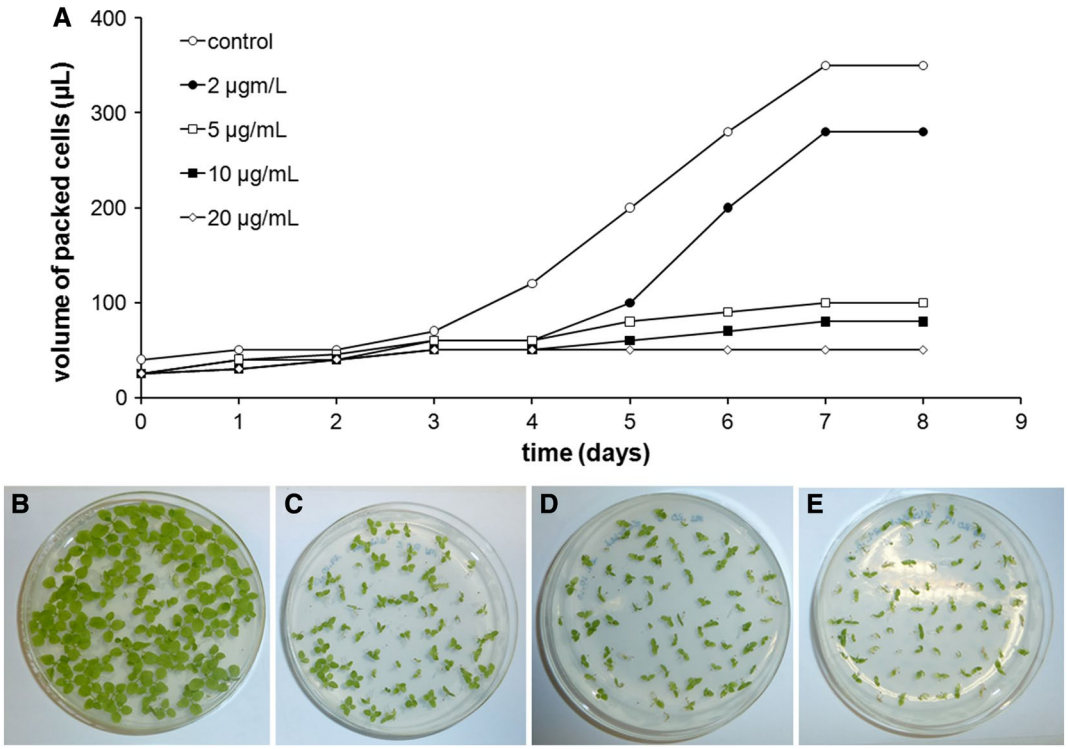

Fig. 2 Determination of a working concentration for *Nicotiana* species treatment with RO 48-8071. **a** Growth curve for *Nicotiana tabacum* BY-2 cells treated with increasing concentrations of RO 48-8071 inhibitor. Results show a representative trial. **b–e** Effect of RO 48-8071 on *Nicotiana benthamiana* seedling growth. *Nicotiana benthamiana* seedlings (15-day-old) were grown in MS medium supplemented with either acetone (**b**) or with 2 µg/mL (**c**), 5 µg/mL (**d**) and 10 µg/mL RO 48-8071 (**e**) dissolved in equal quantities of acetone. The solvent (acetone) alone had no effect on seedling growth

stigmasterol and sitosterol is much more pronounced. Interestingly, there was no further accumulation of squalene and oxidosqualene. In the GLC separation, the treatment for 24 h led to the appearance of some new peaks with a Rt lower than that of campesterol, one of which corresponds to oxidosqualene (Fig. 3a). Those peaks largely disappeared after prolonged (6 days) treatment (Fig. 3b) but, at this stage, the peak corresponding to 24-methylenecholesterol became prominent (Fig. 3b).

Gene Expression Analysis

After 6 days of treatment, the expression of *NtCAS1* (Fig. 3c) increased 2.5 fold in the BY-2 cells. In contrast, *NtSMT2.2* expression decreased 50 % under the same conditions (Fig. 3d). *NtHMGR1* (Fig. 3e) and *NtHMGR2* (Fig. 3f) expression decreased to about one-third of the initial level.

Sterol and Gene Expression Analysis in amiRNA CAS1-Silenced BY-2 Cells

To further examine the role of CAS in BY-2 cells, an inducible (artificial) silencing technique (amiRNA) was applied. Two corresponding transformed cell lines were selected from calli (lines 1.8 and 4.8). Synthesis of amiRNA against *NtCAS1* was induced from pER8.1 vector by estradiol. Controls (transformed cells bearing the empty vector pER8.1) were also treated with estradiol, followed by sterol analyses after 24 h (Fig. 4a, b) and 6 days of treatment (Fig. 4d, c), indicating a small accumulation of oxidosqualene after 24 h but few other changes, with none in line 1.8. However, line 4.8 did show a small decrease in campesterol and stigmasterol content. These differences were visible after 24 h and 6 days. Levels of estradiol-induced mRNA were quantified by qRT-PCR 15 h, a time period previously experimentally established as a standard condition for working with the estradiol-inducible pER8.1 system. In both cell lines about 35 % reduction in mRNA encoding NtCAS was observed (Fig. 4e). Partially blocking the expression of *NtCAS1* led to a 40 % increase in *SMT2.2* expression (Fig. 4f). Overall, sterol content was mostly unchanged, suggesting a decreased flux through the sterol pathway due to the reduction in *NtCAS1* gene expression. However, this was almost totally overcome at the end of the cell culture growth curve.

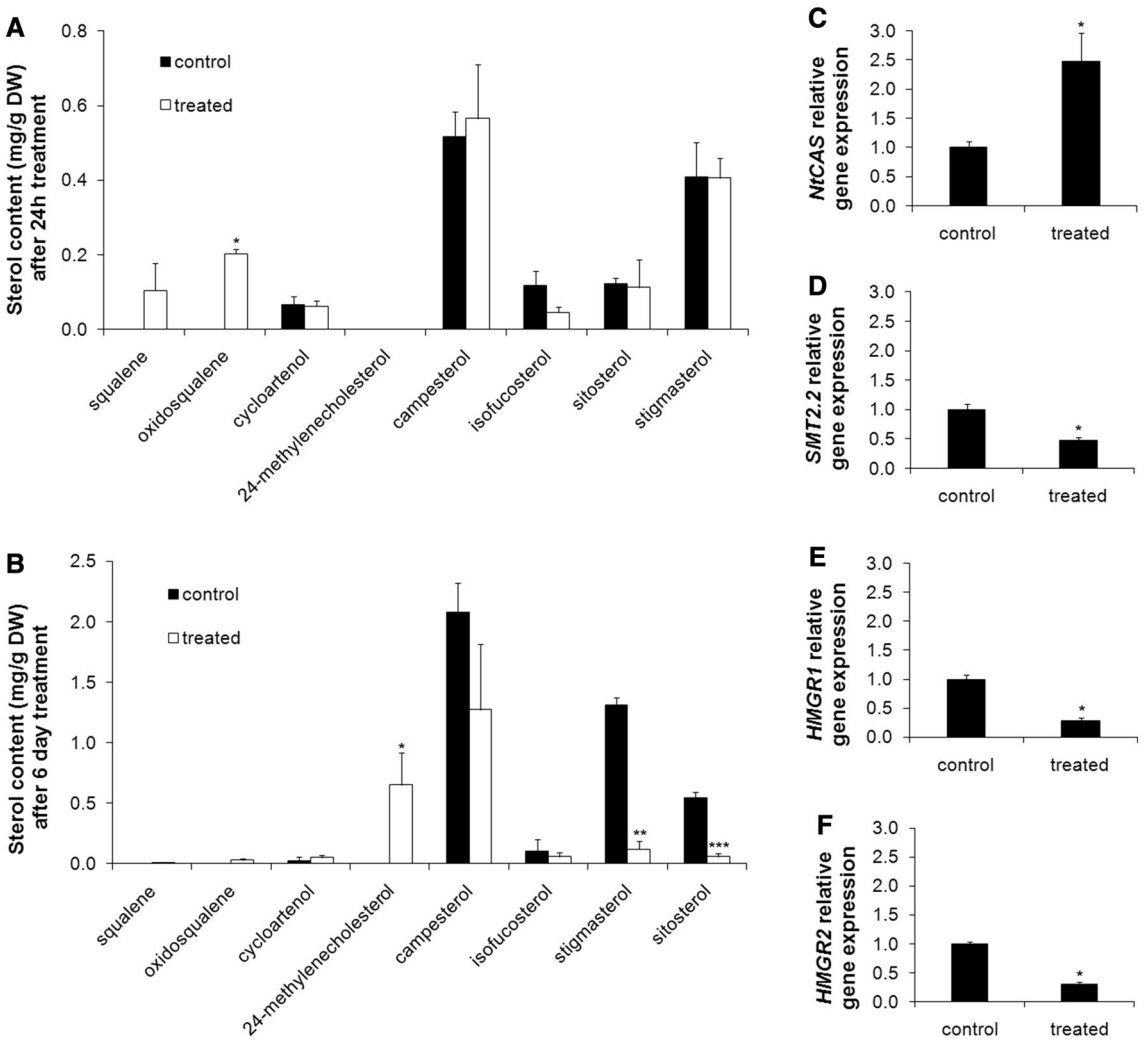

Fig. 3 RO 48-8071 effect on sterol content of BY-2 cells. *Nicotiana tabacum* BY-2 cell suspensions were treated 1 day after sub-culture with or without 2 µg/mL of RO 48-8071. Sterol contents were measured 24 h after the treatment (at day 2 after sub-culture) and after 6 days of treatment, at the end of the growth curve (day 7 after sub-culture). Gene expression levels were measured by qPCR after 6 days of treatment (day 7). Experiments and measurements were repeated three times. Statistically significant changes are indicated (*asterisk*) and *p* values for *t*-student tests are indicated in Table S1. **a** Sterol content after 24 h of treatment (day 2). **b** Sterol content after 6 days of treatment (day 7). **c** *NtCAS1* expression levels at day 7. **d** *NtSMT2.2* expression levels at day 7. **e** *NtHMGR1* expression levels at day 7. **f** *NtHMGR2* expression levels at day 7

Treatment and Analysis of Arabidopsis Cell Suspensions and Seedlings

A green Arabidopsis cell suspension was treated with RO 48-8071 at 2 and 5 µg/mL, to determine the effects of CAS inhibition and RO 48-8071 selectivity on the synthesis of non-sterol triterpenes like lupeol and α-/β-amyrin, which do not occur in BY-2 cells. In 5 µg/mL inhibitor the cells appeared bleached, and there was a marked decrease in cell density (Fig. 5b). There was a drastic reduction in the content of end-of-chain phytosterols (Fig. 5a).

When Arabidopsis seedlings were treated with 5 µg/mL of RO 48-8071, phytosterol content was altered in a similar way to in the BY-2 cells; oxidosqualene accumulated, while sitosterol and stigmasterol contents decreased by more than 50 %. No major differences were observed in lupeol and α/β amyrin content (Fig. 6). A cytotoxic effect in the first pair of true leaves became visible in 2 µg/mL inhibitor, as bleaching and loss of the typical leaf size and morphology (Fig. 6b).

Discussion

Different Approaches to Analysis of Phytosterol Biosynthesis

Although the role of phytosterol biosynthesis in vital processes of plants is generally understood [1–10], the

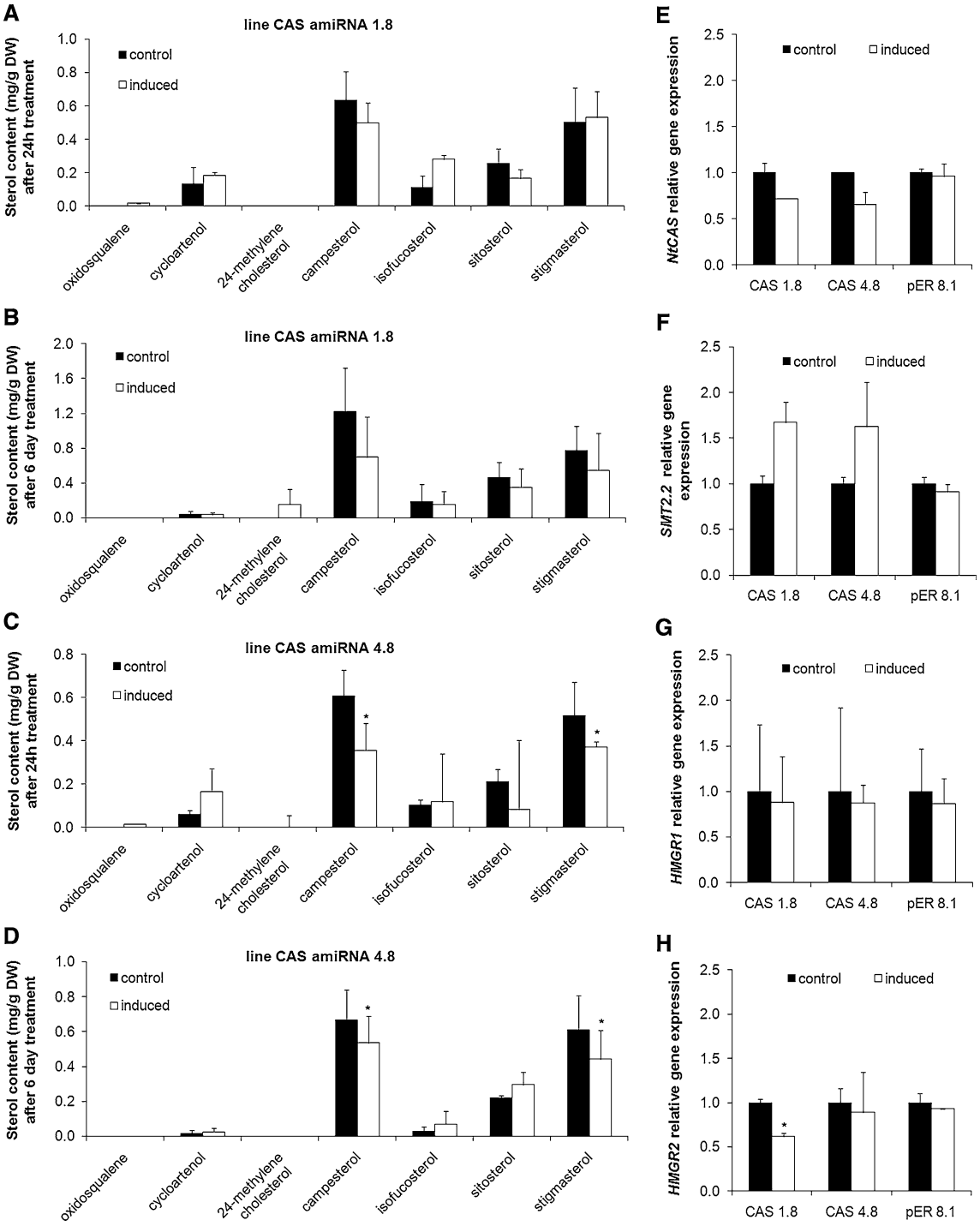

Fig. 4 *NtCAS* gene silencing via inducible amiRNA in BY-2 cells. Two *Nicotiana tabacum* BY-2 lines, independently transformed with an inducible amiRNA construct against *NtCAS* (CAS 1.8 and CAS 4.8) were treated 1 day after sub-culture with or without 5 μM estradiol. Sterol contents were measured 24 h after the treatment (at day 2 after sub-culture) and after 6 days of treatment, at the end of the growth curve (day 7 after sub-culture). Gene expression levels were measured by qPCR after 6 days of treatment (day 7). Experiments and measurements were repeated three times. Statistically significant changes are indicated (*asterisk*) and *p* values for *t*-student tests are indicated in Table S2. **a**–**b** Sterol content in line 1.8 after 24 h (day 2, **a**) or after 6 days of treatment (day 7, **b**). **c**–**d** Sterol content in line 4.8 after 24 h (day 2, **c**) or after 6 days of treatment (day 7, **d**). **e**–**h** Gene expression levels at day 7 for *NtCAS* (**e**), *NtSMT2.2* (**f**), *NtHMGR1* (**g**), and *NtHMGR2* (**h**). Changes in expression in a BY-2 control line (transformed with the empty vector, pER8.1) are also shown

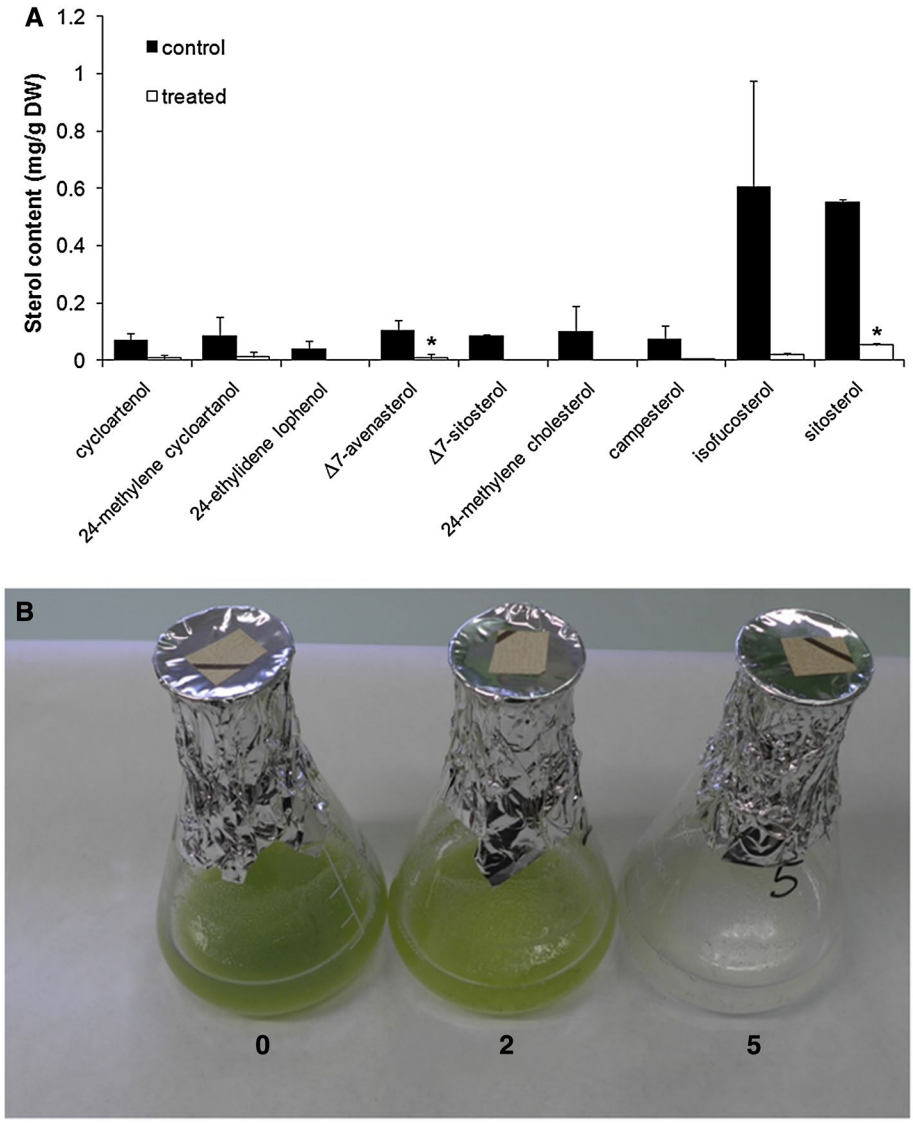

Fig. 5 Effect of RO 48-8071 treatment on Arabidopsis MMD2 cell suspensions. *Arabidopsis thaliana* MMD2 cells suspensions were grown in modified MS medium supplemented with either acetone or 5 µg/mL RO 48-8071 dissolved in equal quantities of acetone. The solvent (acetone) alone had no effect on the cell suspension growth. **a** Sterol content in cell suspensions measured after 3 days of treatment. Experiments and measurements at 5 µg/mL RO 48-8071 were repeated twice. Statistically significant changes are indicated (*asterisk*) and *p* values for *t*-student tests are indicated in Table S3. **b** Effect on cell suspension growth at 0, 2, and 5 µg/mL RO 48-8071

regulatory details are still obscure. In this contribution, we focused our attention on one key enzyme in the pathway, CAS [14, 15, 23, 43–45], and on its regulatory role in the well-established tobacco BY-2 cell system [33, 34]. Admittedly, observations on cell cultures may not always hold true for intact plants. Nonetheless, significant conclusions at the level of biochemistry, stress reactions, and so forth may be drawn. Inhibition of pathways, be it by chemical inhibitors, gene silencing or random mutagenesis, followed by targeted selection, can yield useful information.

The values show that cells coming from the stationary phase and diluted into new medium possess a stock of end-of-chain sterols like campesterol (actually a mixture of (Δ^5) campesterol together with (Δ^5) 22-dihydrobrassicasterol according to [42]), stigmasterol, and sitosterol. The treatment by the inhibitor led to some accumulation of squalene and its epoxide, an effect that became less evident after a 7-day culture period. However, we see now the appearance of 24-methylenecholesterol, whereas the intermediates go below the level of clear detection. The relative decrease in stigmasterol and sitosterol, less pronounced with campesterol, is clearly a consequence of inhibitor treatment.

Effects of RO-48-8071 Treatment on Expression of Genes Encoding *CAS1* and *SMT2*

The observations above suggest some effect at the level of SMT2 (see Fig. 1). Two methylation steps occur within the sterol pathway. The first one, catalyzed by SMT1, occurs right after cycloartenol synthesis and adds a methyl group at position 24 onto this molecule. The second step, catalyzed by SMT2, converts the C24 methyl group into a C24 ethyl group. The stimulation of the *CAS1* gene in response to chemical inhibition of the CAS enzyme, combined with

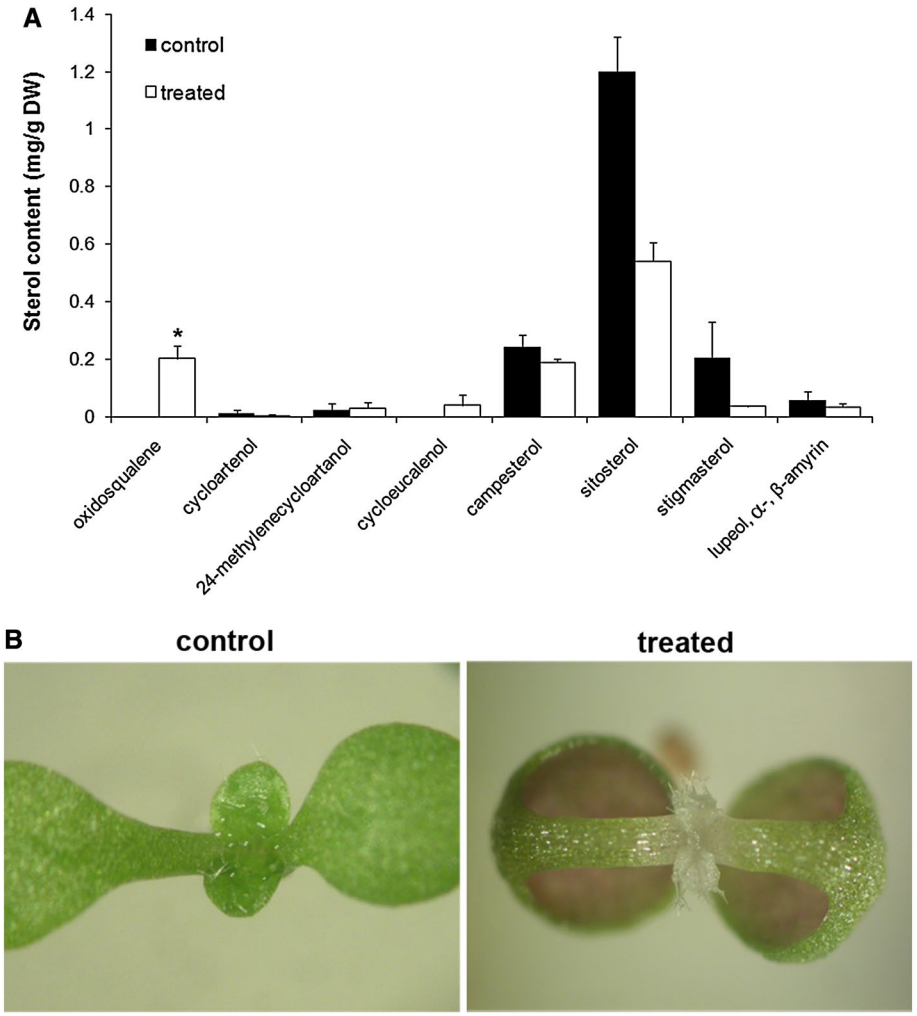

Fig. 6 Effects on Arabidopsis seedling growth after RO 48-8071 treatment. *Arabidopsis thaliana* seedlings were grown in MS medium supplemented with either acetone or with 5 µg/mL RO 48-8071 dissolved in equal quantities of acetone. The solvent (acetone) alone had no effect on seedling growth. **a** Sterol content in seedling measured after 3 weeks of treatment. Experiments and measurements at 5 µg/mL RO 48-8071 were repeated twice. Statistically significant changes are indicated (*asterisk*) and *p* values for *t*-student tests are indicated in Table S4. **b** Effect on Arabidopsis seedling growth after 10 days of treatment at 0 and 5 µg/mL RO 48-8071

the concomitant reduction in *SMT2* expression confirms that the ratio of campesterol to sitosterol is controlled by *SMT2* [46], while the total content of sterols might be regulated at the level on *SMT1* [47]. Final confirmation is challenging due to low levels of cells following inhibitory treatments, and low activities of enzymes downstream of HMGR in membrane extracts of BY-2 cells, and the difficulty in obtaining the enzyme substrates for in vitro enzyme assays.

Effects on the Expression of Genes Encoding HMGR Isoenzymes 1 and 2

The drop in *HMGR1* and *HMGR2* expression under inhibitory conditions agrees with previous data using RO 48-8071. The inhibitor was first described as a drug that reduced cholesterol levels in mammals, without triggering HMGR over-expression [35]. The accumulation of squalene, after inhibition of squalene epoxidase by terbinafine, even as lipid droplets in the cytoplasm of BY-2 cells, was not toxic [32]. When these two enzymatic steps (squalene synthase and squalene epoxidase), which precede the CAS-catalyzed reaction, were inhibited in BY-2 cells, the expression of their corresponding genes was stimulated [32]. In the case of CAS inhibition, a minor accumulation of oxidosqualene might exert a negative effect on cell division and growth and even on chloroplast development [23]; thus a feedback loop triggering over-expression of HMGR would lead to a further accumulation of oxidosqualene and probably more pronounced toxic effects.

Interpretation of Gene Expression Analysis in amiRNA Silenced BY-2 Cells

The reduction in *CAS1* expression in BY-2 cells transformed with amiRNA was only moderate (about 35 %), which at first sight appears disappointing. However, this reduction may reflect the failure to completely silence genes encoding enzymes in the MVA pathway and in phytosterol biosynthesis. Apparently, the small interfering RNA system depends on an intact MVA and sterol

biosynthesis [9]. When two miRNA action-deficient (*mad*) mutants (*mad3* and *mad4*) from Arabidopsis were characterized and the underlying genes identified, it was found that *MAD3* encodes HMGR1, the key enzyme in the early MVA pathway [9]. The related gene *MAD4* encodes sterol C-8 isomerase that acts downstream of the MVA pathway in phytosterol biosynthesis. ARGONAUTE (AGO) protein complexes are controlled by miRNAs to regulate expression of complementary RNAs via base pairing [9]. In Arabidopsis, AGO1 acts as a peripheral membrane protein that apparently needs sterols for membrane association [9]. Thus, achieving a higher level of amiRNA-based silencing of a *CAS* gene beyond the degree we observed might be difficult. This scenario might not hold true for other organisms, like the protozoan *Trypanosoma brucei*. In this organism, whose sterol biosynthesis depends on ergosterol, inducible amiRNA silencing of *TbSMT* (coding for C24-β-methyl transferase) and of *TbSDM* (coding for sterol 14α-demethylase) was shown to be more efficient than in our system, albeit not complete [30]. Nonetheless, some effects on sterol accumulation in estradiol-induced cells versus vector BY-2 control cells could be seen. The total disappearance of oxidosqualene, the minimal if any effect on *CAS1* expression, accompanied by nearly normal levels of cycloartenol, could be interpreted to mean that CAS is not rate-limiting under those conditions and/or that it is subject to regulatory mechanisms which are, as yet, unknown.

Experiments with a Green Arabidopsis Cell Suspension, Arabidopsis, and *N. benthamiana* Seedlings

Green Arabidopsis cell suspension cultures and intact Arabidopsis and *N. benthamiana* seedlings behaved similarly to the BY-2 cell suspensions. *N. benthamiana* seedlings showed a delay in seed germination as well as bleaching as the concentration of inhibitor increased. In Arabidopsis intact seedlings, the bleaching effect at the early seedling stage is in perfect accordance with observations made with *cas1* Arabidopsis mutants [23], where terminal phenotypes included an arrest of meristematic activity, followed by necrotic death. Germinating seed contained sufficient phytosterols to allow a ± normal development of cotyledons, but when the de novo synthesis was inhibited by RO 48-8071, aberrant growth resulted. This was accompanied by a cytotoxic effect, perhaps induced by oxidosqualene, which impeded the development of chloroplasts in Arabidopsis *cas1* mutants [23]. Interestingly, no significant changes were observed in lupeol and amyrin content in inhibitor-treated Arabidopsis seedlings. This RO 48-8071 specificity towards the oxidosqualene cyclase, indicates that this enzyme is directly involved in sterol biosynthesis for CAS in plants and LAS in animals. Green Arabidopsis cell suspension cultures were more drastically affected than in BY-2 cells or whole seedlings, with severe reductions in sterol content at all assayed concentrations. This might indicate a stronger dependence on sterol biosynthesis in this type of cell suspension or an increased sensitivity to chemical inhibition.

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