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Methyl jasmonate induced accumulation of kalopanaxsaponin I in *Nigella sativa*

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ABSTRACT

Hydroponically cultivated *Nigella sativa* L. plants treated with methyl jasmonate (MeJA) showed a twelve-fold increase in levels of the monodesmosidic triterpene saponins α-hederin and kalopanaxsaponin I (KsI) in the leaves. We will demonstrate that these two saponins accounted for approximately 10% of the dry plant matter, of which 93% was KsI and 7% α-hederin. To address the molecular basis of saponin induction by MeJA, we cloned and characterized the *β-amyrin synthase* gene (*NsβAS1*) encoding one of the key enzymes in triterpene saponin biosynthesis. As expected, *NsβAS1* transcription was induced by MeJA and led to the production of β-amyrin when over-expressed in yeast.

1. Introduction

"There is healing in black cumin for all diseases except death" were words of the prophet Muhammad in his Book of Hadeth (Al-Bukhari, xxxx) reflecting the medicinal impact of *Nigella sativa* L. in the Arabic folk medicine. Its outstanding tradition becomes obvious from seed remains found in jars at a late Bronze Age shipwreck (~ 1300 B.C.) at the south west coast of Turkey (Nesbitt, 2006). Today, *N. sativa* is still cultivated mainly for seed and oil production in the Middle East and Northern Africa. In recent years, comprehensive studies with crude seed extracts revealed a wide spectrum of valuable pharmacological properties including e.g. anti-cancer, anti-diabetic and anti-inflammatory activities (for reviews see Ghedira, 2006; Ramadan, 2007).

Several substances that could be of pharmacological interest have been isolated from *Nigella* seeds (Ansari et al., 1988; Taskin et al., 2005; Padhye et al., 2008). Subsequently, detailed biochemical analysis of seeds from the closely related species *Nigella glandulifera* identified the two triterpene saponins, α-hederin and kalopanaxsaponin I (KsI), for being responsible for the anti-cancer activity (Tian et al., 2006). However, the precise mechanism of action remains elusive.

Triterpene saponins occur in plants and some marine organisms (Hostettmann and Marston, 1995) and their involvement in plant defense has been demonstrated (Papadopoulou et al., 1999). The saponins of *N. sativa* supposedly have a similar function. Triterpenes are generally considered to be synthesized through the cytosolic mevalonic acid pathway of isoprenoid biosynthesis (Hostettmann and Marston, 1995). For the model organism *Medicago truncatula* it has been shown that in the entire biosynthetic pathway from IPP (isopentenyl pyrophosphate) to saponins genes are up-regulated by methyl jasmonate (MeJA; Suzuki et al., 2002; Achnine et al., 2005). Moreover, also in the medicinal plant *Panax ginseng* saponin biosynthetic genes (Fig. 1) *squalene synthase* (*SQS*), *squalene epoxidase* (*SQE*), *β-amyrin synthase* (*βAS*), *cytochrome P450s* (*CYP450s*) and *glycosyltransferases* (*GTs*) were found to be induced upon treatment with MeJA leading to increased saponin levels (Choi et al., 2005). In higher plants jasmonate and its methyl ester MeJA are regarded as phytohormones that modulate flowering and senescence and trigger plant stress and defense reactions (Wasternack and Parthier, 1997; Creelman and Mullet, 1997).

MeJA-inducible plant cell suspension cultures for the production of saponins have been already established for e.g. *P. ginseng* (Lu et al., 2001) and *Glycyrrhiza glabra* (Hayashi et al., 2003),

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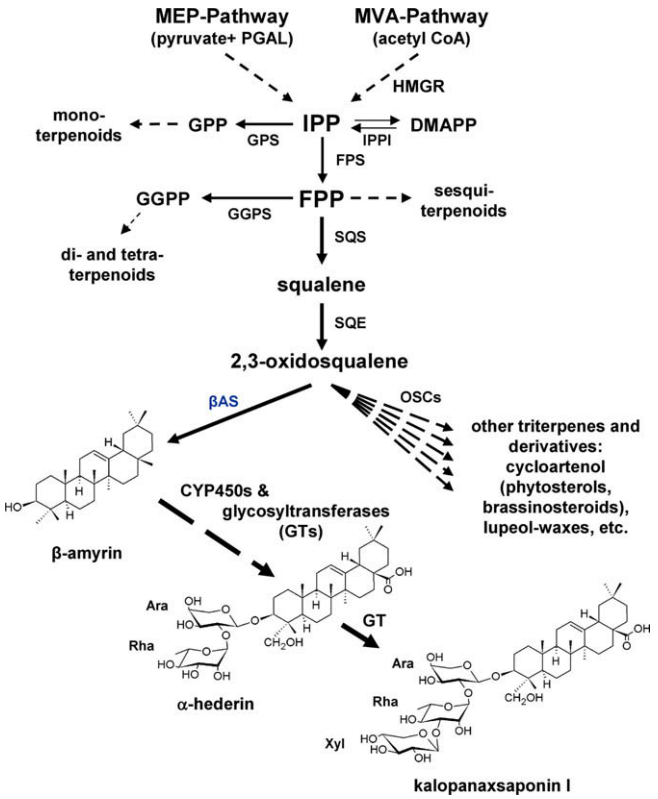

Fig. 1. Schematic representation of the terpenoid biosynthetic network in *N. sativa* with special emphasis on the biosynthesis of monodesmosidic triterpene saponins. Abbreviations are explained in the introduction except: Ara: Arabinose, Rha: Rhamnose, Xyl: Xylose; OSC: oxidosqualene cyclase, HMGR: Hydroxymethylglutaryl CoA reductase, MEP: Methylerythrytol phosphate, PGAL: Glyceraldehyde 3-phosphate, DMAPP: Dimethylallylpyrophosphate, IPPI: IPP isomerase, GPP: Geranylpyrophosphate, GPS: GPP synthase, FPP: Farnesylpyrophosphate, FPS: FPP synthase, GGPP: GeranylGPP and GGPS: GGPP synthase.

however, neither α-hederin nor KsI could be detected in a *N. sativa* cell suspension culture treated with the same phytohormone (M. Scholz, unpublished results).

Here we report for the first time that the occurrence of the monodesmosidic saponins α-hederin and KsI in *N. sativa* is not restricted to the seeds and that substantial amounts can be isolated from leaves. We developed a hydroponic system to cultivate *N. sativa* under controlled conditions. Using this cultivation system, we will show that the treatment with MeJA leads to a twelve-fold increase of α-hederin and KsI in *N. sativa* and that KsI is the dominating saponin. Furthermore, we cloned the *β-amyrin synthase* gene (*NsβAS1*) of the triterpene saponin biosynthetic pathway from *N. sativa*. *NsβAS1* was induced by MeJA and produced β-amyrin when expressed as a recombinant protein in yeast.

2. Experimental

2.1. Cloning of NsβAS1 and expression analysis

The *NsβAS1* gene was obtained as follows: *N. sativa* total leaf RNA was prepared using QIAzol lysis reagent (Qiagen, Hilden, Germany) according to the manufacturers' instructions and subsequently sent to Eurofins/Medigenomix (Munich, Germany) for the construction of a representative λ TripleEx2 cDNA library. For library screening, a 900 bp *NsβAS1* cDNA fragment was first amplified by 3'-RACE using total leaf RNA from seedlings harvested 3 days after MeJA treatment, the degenerate primer NsβAS1-1 (5'-GAY CAR GAY CAY GGG TGG CAG-3′) and the 3' RACE adapter primer (GAC TCG TGT GGA CAT CGA) that corresponded to the backward RACE primer (GAC TCG TGT GGA CAT CGA TTT TTT TTT TTT TTT TT). Approximately 3 million plaques of the amplified library were screened; radioactive Klenow-labelling of the DNA probe was performed with the ReadiPrime II Kit (GE Healthcare, Munich, Germany). Hybridizations of plaque lift offs were carried out in 6X SSC, 0.1% SDS, 0.1% Ficoll-400, 0.1% PVP-15 at 53 °C in a shaking water bath. The genomic sequences for *NsβAS1* were obtained by PCRs on genomic DNA with primers deduced from the *NsβAS1* full-length cDNA clone. All clones were subcloned into the pCR®II-Topo® vector (Invitrogen, Karlsruhe, Germany) and sequenced using established protocols (Sanger et al., 1977).

For gene expression analysis, *NsβAS1* was amplified from total RNA by reverse transcription using Superscript II (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations. The following primers were used for RT–PCR expression analyses: Nsβas1a (5'-GGG TGG CAA GTC TCA GAT TGT -3') and Nsβas1b (5'-CGA TAC TCT CCA AGA GCC CAC -3') for *NsβAS1* amplification, NsGapDHfw (5'-TCG GGA TCA ACG GGT TTG GAA G-3') and NsGapDHbw (5'-CTC TCC AGT CCT TGC TTG ATG GTC-3') for detection of *GAPDH*. The software used for quantification of RT–PCR products was ImageJ (http://rsbweb.nih.gov/ij/).

2.2. Hydroponics cultivation system and MeJA treatment

N. sativa seeds were obtained from Wildacker (Wolmersdorf, Germany). After germination of seeds on wet paper towels in the dark, seedlings were further cultivated at 21 °C under a long day regime (16:8 h light–dark cycles) for 10 days. In order to produce plantlets that could be transferred into the hydroponic cultivation system after germination the light intensity was set to 50 μmol m^{-2} s^{-1} during this time to reduce shoot growth and to promote root elongation. Seedlings were transferred to polypropylene meshes on plastic boxes (10 × 10 × 3.7 cm, 9 seedlings per box) containing 175 ml half strength modified Hoagland liquid medium (2.5 mM KNO_3, 2.5 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 500 μM KH_2PO_4, 200 μM FeEDTA, 92.5 μM H_3BO_3, 18.3 μM $MnCl_2$, 1.5 μM $ZnSO_4$, 0.64 μM $CuSO_4$, 0.24 μM Na_2MoO_4; Hoagland and Arnon, 1950). The pH was adjusted to 5.8. The transferred seedlings were cultivated for two weeks under a long day regime (16:8 h light–dark cycles, 200 μmol m^{-2} s^{-1}, 23 °C) under transparent covers to reduce evaporation. Induction experiments were carried out by adding MeJA (100 μM) directly to the half strength Hoagland medium. For subsequent experiments total aerial biomass of the seedlings was collected, immediately frozen in liquid nitrogen and stored at −80 °C until use. Plantlets from single pots were treated as individual samples. Triplicate samples were taken for each data point. During pre-culture and MeJA induction of *N. sativa* in hydroponics the culture medium was changed every 2 and 1 days, respectively.

2.3. Extraction of saponins and quantification by HPLC

Plant material stored at −80 °C was freeze dried and ground to a fine powder. 500 μl of solution of the internal standard (IS, see below, Glycyrrhizinic acid; Carl-Roth GmbH, Karlsruhe, Germany) was added to a reaction vessel and evaporated to dryness (Concentrator 5301, Eppendorf, Germany). Afterwards 5 mg of the powder was transferred into this vessel and extracted 5× using 1 ml aqueous EtOH (90%). The first extraction was carried out in an ultrasonic bath for 30 min and the remaining extractions by vortexing for 1 h, respectively. The extracts were combined and the solvent was evaporated. The dry residue was dissolved in 500 μl 80% aqueous MeOH containing 0.02% TFA and the solution was purified through a 0.2 μM filter.

Quantification was performed on an Ettan HPLC System (GE Healthcare, Munich, Germany) using an YMC ODS-A reversed

phase column (250 mm × 4.6 mm, 5 μM). Elution of compounds was monitored at 214 nm (α-hederin and KsI) and 254 nm (IS). The mobile phases consisted of 0.75% TFA in H_2O (A) and MeCN (B). Injection volume was 20 μl. Saponins were separated at ambient temperature (25 ± 1.5 °C) using a triphasic linear gradient program: 35–44% B in 9 min, 44–52% B in 18 min and 52–100% B in 5 min. The flow rate was kept at 0.6 ml/min.

The IS stock solution of 500 μg/ml was prepared in a volumetric flask in 80% aqueous MeOH containing 0.02% TFA. The IS solution was used as solvent for a saponin stock solution containing 2 mg/ml α-hederin (Phytolab, Vestenbergsreuth, Germany) and subsequent serial dilutions (1, 0.5, 0.25, 0.1, 0.05 and 0.01 mg/ml). These calibration solutions were injected in triplicate. A calibration plot of the area ratios of α-hederin/IS *vs*. α-hederin concentration was constructed ($y = 1.07905x + 0.00579$, $R^2 = 0.99997$) for α-hederin quantification. As expected and verified by HPLC, KsI and α-hederin showed the same molar absorption properties. Therefore, calculation of KsI content was carried out based on the α-hederin calibration taking in account the different molecular weights of both compounds. KsI was isolated and identified by HPLC/MS according to established protocols (Taskin et al., 2005).

2.4. Cloning and expression of *NsβAS1* in yeast and β-amyrin quantification

The coding region of *NsβAS1* was amplified by PCR using the primers AAA AAG ATC TAA ATG TGG AAG CTG AAG ATA GCT GAT GCA AC (*Bgl*II restriction site is underlined) and AAA AGC GGC CGC CTA ACT TGA TAA ACA CTT CCG GCG (*Not*I restriction site is underlined). After digestion with *Bgl*II and *Not*I the PCR fragment was inserted under the control of the ADH promoter into the corresponding restriction sites of PADNX, which is a derivative of PADNS (Colicelli et al., 1989). The integrity of the resulting constructs was verified by sequence analysis. Empty vector control (PADNX) and PADNsβAS1 constructs were transformed into the Y187 yeast strain (Clontech, Heidelberg, Germany) by the Li–Acetate/PEG method (Gietz and Woods, 2002). Transgenic yeast strains were selected on SD-Leu medium. For the production of β-amyrin 50 ml selective medium was inoculated in Erlenmeyer flasks with single transformed colonies and grown in a shaking incubator at 30 °C for 48 h. Saturated cultures were collected by centrifugation and the pellet was dissolved in 2 ml of 10% KOH in MeOH. Saponification was carried out in glass vials for 1 h at 80 °C. Subsequently, 2 ml ultra pure water and 2 ml of hexane were added and mixed for 1 min. After separation, the hexane phase was collected and dried in a vacuum exsiccator. Samples were silylated with 100 μl MSTFA (Fluka, Buchs, Switzerland) for 30 min at 60 °C and then diluted with 400 μl iso-octane (Sigma–Aldrich, Munich, Germany). An Agilent GC6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled with a Quattromicro-GC (Waters-Micromass, Manchester, UK) mass spectrometer was used for sample analysis.

1 μl of sample was injected in the split mode (1:10) and separated on an HP-5 GC-column (30 m length, 0.25 mm ID, 0.25 nm film thickness). The injector temperature was set to 280 °C. Helium was used as a carrier gas at a constant flow of 1.3 ml/min. The column temperature program was as follows: initial temperature was kept for 2 min at 50 °C, then increased to 300 °C at 15 °C/min. The mass spectrometer was set to unit resolution. In scan mode the range 37 to 700 *m/z* was registered within 0.3 s. Selected ion recording (SIR) was measured on the characteristic ion of β-amyrin-TMS 218.3 *m/z* with a dwell time of 0.2 s (cycle 0.38 s).

Quantification of β-amyrin was done with external calibration in SIR-mode. Three concentrations (2, 20 and 200 μg/ml) of an authentic standard (Fluka, Buchs, Switzerland) were used to establish the calibration line. The scan spectrum at 21.01 min agreed perfectly with the NIST-library spectrum of ergosterol-TMS. To compare the ergosterol and β-amyrin levels in different preparations (Fig. 4b), aliquots of the silylated samples were injected, the mass chromatograms on 218.3 *m/z* (β-amyrin-TMS) and 363.3 *m/z* (ergosterol-TMS) were reconstructed from scan data and integrated.

3. Results and discussion

3.1. Induction of kalopanaxsaponin I by methyl jasmonate

Initially, we investigated the potential of MeJA to induce α-hederin and KsI biosynthesis in hydroponically cultivated *N. sativa* plants. Therefore, MeJA in a final concentration of 100 μM was added directly to the nutrient solution without damaging the plantlets. In three individual experiments (Fig. 2), we measured the concentrations for α-hederin and KsI at six different time points in MeJA-treated and non-treated plants, respectively. Indeed, α-hederin and KsI biosynthesis was clearly induced by MeJA with highest saponin concentrations seven days after treatment. Within the analysed period a thirteen-fold increase in the amount of KsI was evident with mean values of 7.4 (day 0) to 95 mg per gram of dry weight (mg/g dw) at day 7, whereas accumulation of α-hederin was six-fold ((1.1 mg/g dw (day 0) to 6.7 mg/g dw (day 7)). In total, the concentrations of both monodesmosidic saponins reached approximately 10% of dry matter with KsI concentrations fourteen-fold higher compared to α-hederin. Therefore, we conclude that KsI might represent the end product of saponin biosynthesis in *N. sativa* while α-hederin is the immediate precursor (see

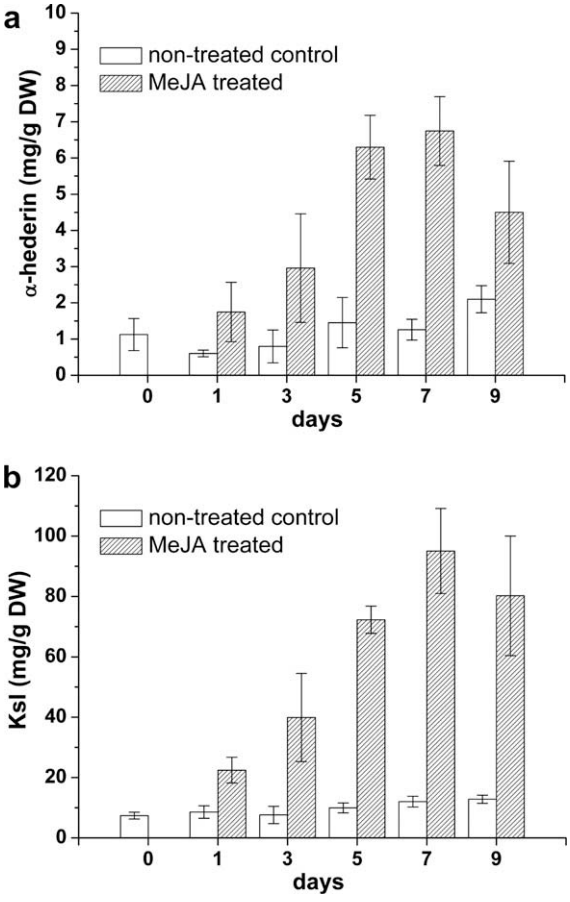

Fig. 2. Amount of α-hederin (a) and kalopanaxsaponin I (b) in aerial tissues of *N. sativa* upon treatment with methyl jasmonate. Non-treated *N. sativa* plants served as controls.

proposed biosynthetic route in Fig. 1). No further increase in the levels of α-hederin and KsI were obtained at time points beyond 7 days. Compared to non-treated controls, MeJA-treated *N. sativa* plants showed at this time point first senescent phenotypes, which might account for the slight decrease in α-hederin and KsI concentrations. In parallel experiments the concentration of α-hederin and KsI was determined in roots from MeJA-treated and non-treated plants. However, none of these saponins could be detected in the corresponding root extracts (data not shown).

Accumulation of triterpene saponins have been reported for different plant tissues including e.g. the primula root (5–10% saponins/dw), the quillaia bark (10% saponins/dw), the licorice root (2–12% glycyrrhizin/dw) and the horse chestnut (up to 13% aescine/dw; Hostettmann and Marston, 1995). Interestingly, similar high concentration of triterpene saponins could be identified in leaves of MeJA-treated *N. sativa* plants – a tissue, which easily can be harvested without employing laborious and cost-intensive extractions techniques.

3.2. Cloning and characterization of a Nigella sativa β-amyrin synthase gene

Next, we addressed the molecular basis of MeJA-mediated saponin induction in *N. sativa* by analysing the expression of one of the key enzymes of triterpene biosynthesis. Therefore, we isolated the *β-amyrin synthase (βAS)* cDNA using degenerate primers covering conserved sequence motifs of known *βAS* genes (for details in cloning see the Section 2). Employing this strategy, a 900 bp cDNA fragment was amplified and shown to encode the carboxy-terminal part of a *β-amyrin synthase* by sequence analysis. Subsequently, a *N. sativa* leaf-specific phage λ cDNA library was screened with the radioactive-labelled 900 bp fragment at low stringency in order to obtain the *βAS* full-length clone. In total, 102 positive clones were identified during the individual screening rounds, which turned out to contain either partial or full-length sequences of the same *βAS* gene of *N. sativa*. Based on this sequence information, the corresponding genomic clone was obtained by PCR (see Section 2). Comparison of the cDNA sequences with the genomic clone revealed the presence of a 2286 bp open reading frame, interrupted by 17 introns, encoding a ~87 kDa protein (Fig. 3a). The resulting cDNA clone was designated *NsβAS1* (protein NsβAS1; accession number **FJ013228** for cDNA, **FJ013229** for genomic clone). In addition, computer analysis revealed an approximately 70% sequence identity of the NsβAS1 to *β-amyrin synthases* from other plant species including *Betula platyphylla*, *Bruguiera gymnorrhiza*, *Lotus japonicus*, *M. truncatula*, *Glycyrrhiza glabra, Vitis vinifera*, *P. ginseng*, *Euphorbia tirucalli*, *Polygala tenuifolia* and *Artemisia annua*.

Expression of *NsβAS1* was found in seedlings, leaves, capsules and predominantly in flowers while *NsβAS1* transcription was not detected in roots (Fig. 3b). It should be noted that α-hederin and KsI were found in all tissues except in roots of MeJA-treated and non-treated plants (data not shown). Thus, a strong correlation in the occurrence of *NsβAS1* mRNA, α-hederin and KsI is evident. Interestingly, a comparable linkage between the sites of saponin production and the expression of *β-amyrin synthases* and *β-amyrin specific cytochrome P450* genes was recently described for licorice and oat (Haralampidis et al., 2001; Qi et al., 2006; Seki et al., 2008).

To further address the involvement of NsβAS1 in α-hederin and KsI biosynthesis we examined the expression of the gene in response to MeJA treatment. As shown in Fig. 3c a slight increase in the *NsβAS1* mRNA level first occurred in *N. sativa* leaves 4 h after the addition of MeJA to the nutrient solution. This level remained constant during the analysed timeframe and indicated an approximately four-fold induction of *NsβAS1* transcription compared to the non-treated controls.

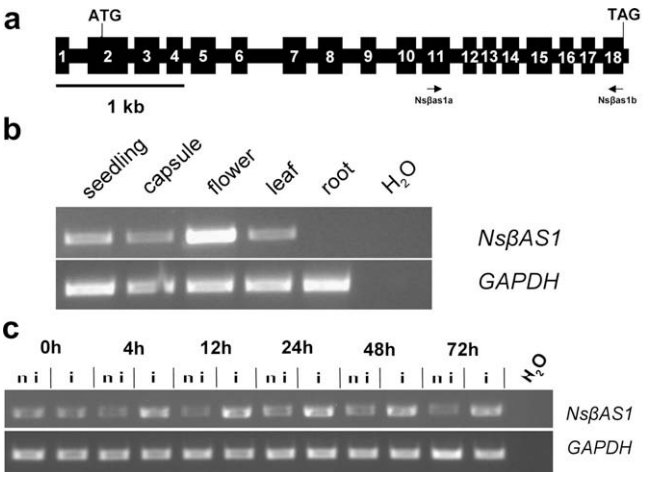

Fig. 3. (a) Genomic structure of *NsβAS1*: numbers refer to the exons identified by comparison of cDNA and genomic DNA sequences. The startcodon (ATG) and stopcodon (TAG) are shown. The positions of primers for subsequent RT–PCR experiments are indicated. (b) Analysis of *NsβAS1* expression in different tissues of *N. sativa* by RT–PCR. The highest mRNA level for *NsβAS1* was detected in flowers and no expression was found in roots. PCR cycle number was 29. (c) Influence of MeJA on *NsβAS1* gene expression. Expression of the *NsβAS1* gene was detected at the different time points by RT–PCR on the basis of total RNA isolated from MeJA-treated (i) or non-treated (n i) *N. sativa* plants. PCR cycle number was 26. In parallel, the quantity and quality of the RNA samples were analysed by monitoring the expression of the housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) (b, c).

It has been reported that MeJA-treatment of suspension cell cultures from *M. truncatula* can induce *βAS* genes up to 50-fold (Suzuki et al., 2005). However, we obtained a relatively weak induction of *NsβAS1* transcription in *N. sativa* plants although α-hederin and KsI biosynthesis was strongly induced. This might be explained by a higher enzymatic activity or stability of NsβAS1 but we cannot exclude that additional *βAS* genes are involved in the biosynthesis of α-hederin and KsI in *N. sativa*. Therefore, functional genomic approaches including RNA interference might be powerful tools to gain further insights into the precise role of NsβAS1 in α-hederin and KsI biosynthesis. However, an efficient and reliable protocol for genetic engineering of *N. sativa* is still missing.

3.3. Expression of NsβAS1 in yeast

To further investigate the function of NsβAS1 in α-hederin and KsI biosynthesis we study its enzymatic activity when expressed as a recombinant protein in yeast. Therefore, the entire coding region of *NsβAS1* was placed under the control of the strong yeast ADH1 promoter and terminator to yield PADNsβAS1. Subsequently, PADNsβAS1 and PADNX (the empty vector control) recombinant yeast strains were analysed for β-amyrin production by GC–MS. Compared to the PADNX control, yeast cells expressing *NsβAS1* (Fig. 4a) produced up to 0.4 mg/l of β-amyrin. Moreover, the synthesis of β-amyrin occurred at the expense of ergosterol, which is the metabolic end product of yeast sterol biosynthesis (Fig. 4b).

In recent years, the production of β-amyrin and further mono- to hexacyclic triterpene intermediates in yeasts by expressing plant *β-amyrin synthases* and other oxidosqualene cyclases was reported (Husselstein-Müller et al., 2001; Phillips et al., 2006; Lodeiro et al., 2007). Thus, yeast appears to be an excellent model system for the functional characterization of this class of enzymes. Taken together, the NsβAS1-mediated production of β-amyrin in yeast together with its MeJA-inducibility strongly argues for the involvement of NsβAS1 in triterpene saponin biosynthesis of *N. sativa*.

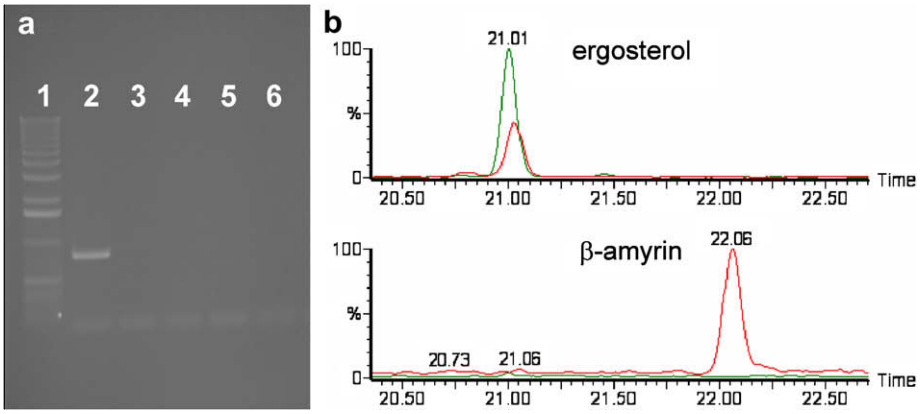

Fig. 4. (a) Detection of *NsβAS1* gene expression in yeast. Total RNA was isolated from either PADNsβAS1 (lane 2 and 3) or PADNX recombinant yeast cells (lane 4 and 5) and subjected to PCR analyses with (lane 2 and 4) or without (lane 3 and 5) addition of reverse transcriptase. Thus, transcription of *NsβAS1* in PADNsβAS1 recombinant yeast cells is indicated. For PCR amplification, the same primer pair as shown in Fig. 3a was used. Lane 1: 1 kb size marker from Invitrogen; Lane 6: PCR water control. (b) Determination of ergosterol (above) and β-amyrin (below) production in yeast extracts prepared from PADNsβAS1 (red) and PADNX (green) recombinant yeast cells. Note that β-amyrin production is achieved at the expense of ergosterol.

NsβAS1 is the first enzyme shown to participate in α-hederin and KsI biosynthesis in *N. sativa* that exclusively takes place in aerial organs.

Genes encoding enzymes responsible for the further metabolization of β-amyrin and intermediates thereof have been cloned and functional characterized. These include CYP450 enzymes (Shibuya et al., 2006; Qi et al., 2006) and glycosyltransferases (Achnine et al., 2005; Meesapyodsuk et al., 2007). In the future it will be interesting to identify further *N. sativa* saponin biosynthetic genes and to analyse if they show a comparable genomic organisation as recently described for the avenacin biosynthetic gene cluster from oat (Papadopoulou et al., 1999; Qi et al., 2004; Mylona et al., 2008).

4. Conclusions

The amount of the triterpene saponin kalopanaxsaponin I was thirteen-fold increased in *N. sativa* treated with methyl jasmonate. A MeJA-inducible *β-amyrin synthase gene* (*NsβAS1*) was identified that led to the production of β-amyrin when over-expressed in yeasts. These characteristics favor NsβAS1 for being one of the key enzymes of the saponin biosynthetic pathway in *N. sativa*.

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