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Quinic Acids from *Aster caucasicus* and from Transgenic Callus Expressing a β-Amyrin Synthase[§]

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Several different classes of secondary metabolites, including flavonoids, triterpenoid saponins and quinic acid derivatives, are found in *Aster* spp. (Fam. Asteraceae). Several *Aster* compounds revealed biological as well as pharmacological activities. In this work, a phytochemical investigation of *A. caucasicus* evidenced the presence of quinic acid derivatives, as well as the absence of triterpene saponins. To combine in one species the production of different phytochemicals, including triterpenes, an *Agrobacterium*-mediated transformation of *A. caucasicus* was set up to introduce *A. sedifolius* β-amyrin synthase (AsOXA1)-encoding gene under the control of the constitutive promoter CaMV35S. The quali-quantitative analysis of transgenic calli with ectopic expression of *AsOXA1* showed, in one sample, a negligible amount of triterpene saponins combined with higher amount of quinic acid derivatives as compared with the wild type callus.

Keywords: Asteraceae, *Agrobacterium*-mediated transformation, quinic acids, triterpene synthase.

Phytochemical investigation of *Aster* spp. evidenced that these species are characterized by different classes of secondary metabolites, including saponins [1,2], caffeolylquinic acids [3], and phenylpropanoids [4]. These compounds are reported to exert valuable pharmacological activities including anticholesterolemic, hemolytic, vaccine adjuvant, immunostimulatory and anticarcinogenic properties [5]. The low concentrations of these compounds in the plant is a major constraint for their exploitation for pharmacological and therapeutic purposes. Methods for enhancing the amount of these compounds in the plant include the transgenic approach based on key enzyme genes of their biosynthesis [6].

It is known that the first committed step in triterpenoid biosynthesis in plants involves the cyclization of 2,3-oxidosqualene to give different triterpene polycyclic skeletons, including α- and β-amyrin and lupeol. These conversions are catalysed by specific oxidosqualene cyclases [7]. To date, more than 30 oxidosqualene cyclase (OSC)–encoding genes have been cloned from several plant species. *AsOXA1*, isolated from *A. sedifolius*, encodes a β–amyrin synthase, as demonstrated by the expression in yeast [8]. The ectopic expression of *AsOXA1* in transgenic barrel medic led to higher accumulation of triterpene sapogenins, as well as enhanced root nodulation [9].

In this work, we report a phytochemical analysis of *A. caucasicus* Willd., which, to the best of our knowledge, has no citations in the literature, showing the presence of quinic acid derivatives and the absence of triterpenoid saponins. To combine in one species the production of different compounds, including triterpenes, we set up an *Agrobacterium*-mediated transformation to introduce the *A. sedifolius* β-amyrin synthase (AsOXA1)-encoding gene in *A. caucasicus*. The quali-quantitative analysis of transgenic callus tissues with ectopic expression of *AsOXA1* showed, in one sample, the presence of triterpene saponins, although in negligible amount, and an accumulation of quinic acid derivatives, similar to the wild type leaf. The other transgenic calli revealed no induction of triterpenes and a low amount of quinic acids, similar to the wild type calli.

The EtOH extract of *A. caucasicus* aerial parts, following repeated chromatographic purification, yielded seven compounds including four quinic acid derivatives, two flavonoids derivatives and one phenolic compound. The structures of quercetin 3-*O*-β-D-glucopyranoside and rutin [10], caffeic acid, chlorogenic acid (**1**), 3,4-di-*O*-caffeoylquinic acid (**2**), 4,5-di-*O*-caffeoylquinic acid (**3**), and 3,4,5-tri-*O*-caffeoylquinic acid (**4**) were elucidated from their NMR and mass spectra, and by comparison with existing data from previous reports [11-14].

To verify the presence of triterpenoid saponins in *A. caucasicus* leaf extract, an LC-MS/MS method was used that in a single step allowed the identification of the components of the crude extract of *A. caucasicus.* The use of this approach allowed us to characterize seven already known compounds, in terms of chromatographic and mass spectrometric properties. The identification of the 3,4- and 4,5-dicaffeoylquinic acid isomers was obtained using their parent ion at *m/z* 515 $[M-H]^-$ and their patterns of fragmentation, as previously reported [15-17]. Moreover, triterpenoid saponins were not detected in this species in contrast to the majority of *Aster* spp. [1,2,18-20].

To assess the possibility of producing triterpene saponins in *A. caucasicus*, leaf explants were transformed with a construct harboring *AsOXA1* cDNA coupled to the cauliflower mosaic virus CaMV35S promoter. Since in the *Aster* genus no transformation method was available, with the exception of *Agrobacterium rhizogenes*-mediated transformation of *A. sedifolius* [21], we established a method for *A. caucasicus* based on optimized tissue culture conditions for callus induction and proliferation after leaf explant transformation with EHA105 disarmed *Agrobacterium tumefaciens* strain. After four weeks, callus tissue was induced on the majority of leaf explants. During proliferation, calli that were grown on Morashige and Skoog (MS) solid medium containing sucrose 3%, 0.94 mg/L 2.4D, 0.18 mg/L BAP and kanamycin (50 mg/L) as selective agent, appeared green and friable. Several kanamycin-resistant calli were isolated and the integration of neomycin phosphotransferase type II (*nptII*) and *AsOXA1* coding sequences was confirmed by PCR analysis (data not shown). The *AsOXA1* transgenic calli did not appear to be different in overall morphology and growth when compared with transgenic calli transformed with empty vector. No shoot regeneration was obtained.

Four independent transgenic calli (Oxa1-1, Oxa1-2, Oxa1-3 and Oxa1-4) were selected and analyzed for *AsOXA1* transcript accumulation by semi-quantitative RT-PCR. Expression of *AsOXA1* transgene was observed in all transgenic calli, whereas no amplification was detected in the non-transgenic calli (both wild type and transformed with empty vector). The RT-PCR analysis of the transgenic calli showed that the transgene was expressed differentially. Oxa1-1 and Oxa1-4 calli showed higher expression levels when compared with the other transgenic calli.

In order to elaborate a quali-quantitative analysis of *A. caucasicus* transgenic calli expressing *AsOXA1*, experiments were performed using an LC-MS system equipped with an ESI source and an ion trap analyzer. All compounds were identified by comparing retention times and *m/z* values in the total current chromatogram with those of the selected standard obtained in the isolation step. The study of the total ion current profile of *A. caucasicus* transgenic callus Oxa1-3 showed the presence of two peaks eluted at 41.56 and 52.10 min. Proton adducted ions of these peaks showed *m/z* values at 473 $[M+H]^+$, and 619 [M+H]+, respectively. The collision induced fragmentation of the ion at 619 $[M+H]^+$ generated a fragment ion at *m/z* 457 (-162 amu), indicating the presence of a hexose. For these peaks, molecular formulas $C_{30}H_{48}O_4$ and $C_{36}H_{58}O_8$ could be hypothesized, respectively [2]. These results led us to assume the presence of triterpene derivatives in negligible amount in transgenic *A. caucasicus* callus Oxa1-3 expressing a β-amyrin synthase. No triterpene derivatives were detected in the other transgenic calli. On the contrary, the transgenic plants expressing *AsOXA1* in *Medicago truncatula* accumulated significantly larger amounts of some triterpene compounds related with the *AsOXA1* expression level found in the leaves [9]. It is noteworthy that *Medicago* spp. produce a complex mixture of pentacyclic triterpene compounds [22]. It is likely that the ectopic expression of *AsOXA1* is able to induce a weak production of triterpenes in *A. caucasicus* because 2,3-oxidosqualene could represent a limiting substrate being involved in the sterol pathway. Indeed, *AsOXA1* encodes an enzyme located at the branch point for sterol (primary) and triterpene (secondary metabolism) biosynthesis. Furthermore, the regulation of this enzyme could be fine-tuned and a strong promoter such as CaMV35S is likely to induce unpredictable effects on *A. caucasicus* metabolism.

The on-line analysis of ethanol extracts of *A. caucasicus* calli showed that quinic acid derivatives occurred in all four *A. caucasicus* transgenic calli. HPLC-UV/PDA and LC-MS chromatograms of total EtOH extracts revealed similar profiles, with the four predominant quinic acid derivatives (compounds **1-4**) found in *A. caucasicus* leaves. In addition, quantitative data showed that compound **3** is the most abundant. However, a different content of quinic acid derivatives was observed among the transgenic calli on the basis of the total absorbance of the chromatograms at 254 and 330 nm that provide a measure of their presence in the analyzed extracts. Oxa1-3 callus showed a higher content of quinic acid derivatives, five times more abundant in respect to the wild type and control callus. The other transgenic calli revealed a content of quinic acids similar to the wild type and the control calli (Figure 1).

It is intriguing that a higher concentration of phenolic compounds occurs in transgenic callus Oxa1-3, the only one producing triterpenes (in low amount) and expressing *AsOXA1* at a low level that apparently conflicts with the biochemical results. However, it is unknown whether cross-regulatory interactions between genes and enzymes involved in triterpene and phenylpropanoid pathways might occur. So far, a large part of the molecular research on the shikimate pathway has been carried out in *Arabidopsis*, and additional studies are required to elucidate the regulation [23]. Alternatively, it could be speculated that the production of quinic acid derivatives in

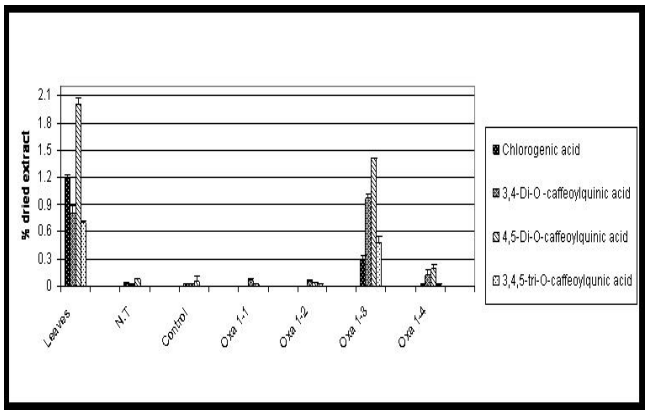

Figure 1: Quinic acid derivative contents of leaves, wild type callus (N.T.), callus transformed with an empty vector (control), and transgenic calli, Oxa1-1, Oxa1-2, Oxa1-3, and Oxa1-4. Values are mean ± standard deviation of three determinations by the HPLC-DAD method.

callus is induced as a general response to the presence of triterpenes, mimicking a stress. Indeed, the phenylpropanoid pathway is induced upon biotic and abiotic stress, such as wounding, UV irradiation, and pathogen attack [24, 25].

In conclusion, four quinic acid derivatives have been isolated from *A. caucasicus*, a species not investigated previously. A method of *Agrobacterium tumefaciens*-mediated transformation of *A. caucasicus* was established whereby obtaining transgenic tissues. Ectopic expression of the β-amyrin synthase (AsOXA1) gene under the control of the CaMV35S promoter was unable to induce a significant amount of triterpenes in *A. caucasicus* callus, but elicited the production of quinic acid derivatives.

Experimental

Plant material: *A. caucasicus*, accession n° 81, from the Botanical Garden of Bayreuth (Germany) was used in this work. To provide leaf explants for callus production and transformation experiments, plants were grown and micro-propagated *in vitro* on a growth regulator-free MS medium [26] supplemented with 3%, w/v, sucrose and 0.8%, w/v, agar (pH 5.7) (MS30). Plants were grown in a climate chamber at 24°C under 14 h of light per day provided by cool white fluorescent tubes at 375 μE/m^2/s. For phytochemical analysis, potted plants, which are perennial, were grown in a greenhouse under natural daylight.

Phytochemical analysis: Dried aerial parts of *A. caucasicus* (100 g) were defatted with light petroleum, then extracted with 80% ethanol (EtOH) to give 6 g of residue. The ethanolic extract was partitioned between *n*-BuOH and H_2O to afford a *n*-BuOH-soluble portion (2.0 g). The *n*-butanol residue (1.6 g) was chromatographed over a column of Sephadex LH-20 (100 x 5 cm) with methanol (MeOH) as eluent (600 mL). A total of 60 fractions were collected (8 mL each) and combined by TLC results on silica 60 F_{254} gel-coated glass sheets with *n*-BuOH-AcOH-H_2O (60:15:25) and $CHCl_3$-MeOH-H_2O (40:9:1), to give 7 pooled fractions (A-G): tubes 1-17 = fr A, tubes 18-23 = fr B, tubes 24-30 = fr C, tubes 30-36 = fr D, tubes 37-40 = fr E, tubes 41-52 = fr F, tubes 53-65 = fr G. Fraction D (128 mg) was submitted to separation by RP-HPLC on a C-18 m-Bondapak column (30 cm x 7.8 mm, flow rate 2.5 mL/min) with MeOH-H_2O (2:3) as eluent to yield pure compounds **2** (5.0 mg, t_R=18 min) and **3** (3.0 mg, t_R=20 min). Fraction E (67 mg) was submitted to separation by RP-HPLC on a C-18 m-Bondapak column (30 cm x 7.8 mm, flow rate 2.5 mL/min) with MeOH-H_2O 1:1 as eluent to yield pure compounds **1** (2.0 mg, t_R=12 min) and **4** (3.0 mg, t_R=14 min), and caffeic acid (4.0 mg, t_R=16 min). Fraction G (105 mg) was purified by RP-HPLC using MeOH-H_2O (2:3) to give quercetin 3-*O*-β-D-glucopyranoside (3.5 mg, t_R= 14 min), and rutin (10 mg, t_R= 11 min). Preparative reversed-phase LC was carried out on an Agilent 1100 series chromatograph, equipped with a G-1312 binary pump, a G-1328A rheodyne injector and a G-1365B multiple wave detector (Agilent, Palo Alto, CA, USA). Separations were performed on a C_{18} column Atlantis 10 x 250 mm (Waters, Milford, MA). Detection responses were measured in terms of peak area using UV detection at a wavelength of 254 nm for caffeoylquinic acids. LC-ESI-MS analysis was performed by using a Thermo Finnigan Spectra System LC coupled with an LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San José, CA, USA). NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker, Rheinstetten, Germany) at 300 K [27].

Identification and purification of compounds 1-4: To ensure the purity of compounds **1**–**4** they were chromatographed by RP-LC using the conditions described above. The purity was confirmed by normalization of peak area, and resulted >95% for compounds **1-4**. Thus, compounds **1-4** were chosen for use as chromatographic standards for calibration curves establishment, showing the essential purity conditions.

Sample preparation: For quantitative purposes, 3 solutions of 1 mg mL^{-1} of an ethanolic extract were prepared. Then a volume of 20 ml of each solution was injected into the chromatographic system. For qualitative purposes, a solution of 1 mg mL^{-1} of the ethanolic extract was prepared and 20 mL was injected into the chromatographic system. A C18 (2.1 x 50 mm, 1.7 μm) column system together with a mobile phase consisting of water (solvent A) and acetonitrile-water (4:6) (v/v) (solvent B) at a constant flow rate of 1 mL min^{-1} was utilized for HPLC separation and analysis. An increasing linear gradient of solvent B was used, starting at 10% of B up to 70% in 7 min. All solvents were filtered through a 0.45 mm Millipore filter before use and degassed in an ultrasonic bath. UV spectra were recorded in the range 190–400 nm, and 254 nm were selected for detection of chlorogenic acid. Stock solutions (1 mg mL^{-1}) of chlorogenic acid derivatives were prepared in MeOH. Five different solutions, containing respectively 6.25, 12.5, 25, 50 and 100 mg mL^{-1} of each standard (external standards) were prepared in H_2O and used for

method development. The calibration curves, for each compound, were made by linear regression by a graph reporting the area ratio of external standard against the known concentration of external standard. The result represents the average of 4 curves performed by 3 injections of each concentration.

LC-MS analysis: An ethanolic extract was analyzed by LC-ESI-MS ''on-line'' using a Thermo Finnigan Spectra System LC coupled with an LCQ Deca ion trap mass spectrometer. Chromatography was performed on a C_{18} column Atlantis (2.1 x 150 mm, 5 μm) (Waters, Milford, MA, USA). A gradient elution was performed by using mobile phase A represented by water acidified with trifluoroacetic acid (0.05%) and a mobile phase B represented by acetonitrile. The gradient started from 0% of eluent B, to achieve 20% of solvent B in 30 min, 30% in 10 min, 50% in 5 min, 70% in another 5 min, and finally 100% in another 5 min. The mass spectrometer operated in the positive ion mode under the following conditions: capillary voltage 37 V, spray voltage 5 kV, tube lens offset 55 V, capillary temperature 300°C, and sheath gas (99.9% nitrogen), flow rate 80 (arb). MS were acquired and elaborated using the software provided by the manufacturer. LC-ESI-MS experiments were carried out by using dependent scanning mode, where the mass spectrometer software made a choice in real time about which ion to fragment and optimised all the parameters to do this based on the charge and on the mass.

Plant transformation and callus production: *CaMV35S::AsOXA1* vector used for *A. caucasicus* transformation was already described by Confalonieri *et al.* [9]. Wounded leaves from *in vitro* grown plants were dipped into an *A. tumefaciens* suspension [optical density 600 nm (OD600) of 1.0] and soaked for 20 min. Inoculated explants were placed on callus induction medium [MS supplemented with sucrose 3%, 0.94 mg/L 2,4D, and 0.18 mg/L BAP] with 500 mg/L kanamycin as selective agent for 72 h in the dark. Subsequently, the infected explants were transferred onto the same medium supplemented with 500 mg/L cefoxatime in the light and subcultured onto fresh medium. Five weeks later, the kanamycin resistant calli were collected and multipled to obtain sufficient quantities of replicated calli for molecular and biochemical analysis. All *AsOXA1* transgenic calli obtained and control with only the neomycin phosphotransferase type II (*nptII*) gene named callus-35S, were maintained and multiplied *in vitro*. All cultures were maintained in a climate chamber at 24°C under 14 h of light per day provided by cool white fluorescent tubes at 375 $\mu E/m^2/s$.

Molecular characterization of transgenic calli: Genomic DNA was extracted from calli of 4 putative As*OXA1* transformants, from callus 35S and control using "DNeasy Plant Mini Kit" (Quiagen, Hilden, Germany). The PCR was carried out in a total volume of 25 μL containing 25 ng of total DNA, by "Taq DNA polymerase" (Invitrogen, CA, USA). The primers used for amplifying the As*OXA1* gene were RT1 (5'-GCCGGTGCATGCTTTTGAAGTCACCG G-3') and RT2 (5'-ACCGAGCCTCTATTAAATCGTTGG CG-3'), the primers for *nptII* gene were NPTIIF (5'-ACGTGCTATTCGGCTATGACTGGG- 3') and NPTIIR (5'-TCAGAAGAACTCGTCAAGAAG GCG–3'), and the 35S promoter primers were 35SF (5'-ATTGAT ATCGTACCCCTACTCCAAAAATG-3') and 35SR (5'-AAGGGTTCCTTATATGCTCAAC-3'). Cycling conditions included 1 cycle at 94°C for 1 min, followed by 35 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 30s, with a final extension step of 10 min at 72°C. To confirm the expression of the transgene, the calli collected from each independent As*OXA1* transgenic event were analyzed, and total RNAs were extracted from 4 transgenic calli and the control by "RNeasy Plant Mini Kit" (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For semi-quantitative analysis, RT-PCR was carried out by "SuperScriptTM One-Step RT-PCR with PlatinumTaq kit" (Invitrogen, CA, USA). The primer set used for RT-PCR amplification was RT1 (5'-GCCGGTGCATGCTTTTGAAGTCACCGG-3') and RT2 (5'-ACCGAGCCTCTATTAAATCGTTGGCCG-3') that amplify a 300 bp transcript. As internal control, the 18S rRNA fragment was amplified by S1 (5'-CACAAACGACTCTCGGCAAC-3') and S3 (5'-CAACCACCACTAGTCGTG-3') primers. Cycling conditions included 1 cycle of 30 s at 50°C, 3 min at 94°C, 40 cycles of 30 s at 90°C, 30 s at 55°C, 30 s at 72°C and with a final extension step of 10 min at 72°C.

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