Plant Physiology and Biochemistry 127 (2018) 287–298

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

# Metabolomics-guided investigations of unintended effects of the expression of the *hydroxycinnamoyl quinate hydroxycinnamoyltransferase* (*hqt*1) gene from *Cynara cardunculus* var. *scolymus* in *Nicotiana tabacum* cell cultures

S.P. Mudau[a], P.A. Steenkamp[a], L.A. Piater[a], M. De Palma[b], M. Tucci[b], N.E. Madala[a,*], I.A. Dubery[a,**]

[a] Department of Biochemistry, University of Johannesburg, P.O. Box 524, Auckland Park, 2006, South Africa
[b] CNR – Istituto di Bioscienze e BioRisorse via Università 133, 80055, Portici, Naples, Italy

ARTICLE INFO

*Keywords:*
Cell culture
Chlorogenic acids
Benzoic acid
*Hqt* gene
Hydroxycinnamoyltransferase
Metabolomics profiling
Phenylpropanoid pathway

ABSTRACT

Chlorogenic acids (CGAs) are phenolic compounds biosynthesized in the phenylpropanoid pathway, with hydroxycinnamoyl quinate hydroxycinnamoyltransferase (HQT) as the key enzyme. Variation of CGAs has been noted in different plants, with globe artichoke (*Cynara cardunculus* var. *scolymus* L.) producing high amounts and a diverse spectrum of CGAs in its leaves. In the current study, the effect of overexpression of the *hqt*1 transgene from globe artichoke in tobacco was evaluated at the metabolome level. Here, metabolomic approaches based on ultra-high performance liquid chromatography coupled to mass spectrometry, together with chemometric models such as principal component analysis and orthogonal partial least square discriminant analysis, were employed to evaluate altered metabolic changes due to *hqt*1 overexpression. CGA profiles (caffeoylquinic acids: 3-CQA, 4-CQA and 5-CQA; *p*-coumaroylquinic acids: 4-*p*CoQA and 5-*p*CoQA; and 4,5-*di*-caffeoylquinic acid) of transgenic tobacco cell cultures were detected at lower concentrations than in the wild type. Interestingly, the cells were found to rather accumulate, as an unintended effect, abscisic acid - and benzoic acid derivatives. The results suggest that insertion of *hqt*1 in tobacco, and overexpression in undifferentiated cells, led to rechannelling of the phenylpropanoid pathway to accumulate benzoic acids. These findings proved to be contrary to the results shown elsewhere in leaf tissues, thus indicating differential metabolic control and regulation in the undifferentiated cell culture system.

## 1. Introduction

Phenolic acids originating from L-phenylalanine (phenylpropanoids) are members of a widely distributed group of secondary metabolites, and contain an aromatic ring with hydroxyl functional group(s) (Le Roy et al., 2016). These compounds include two main classes that can be distinguished based on the structures: benzoic acid (BA) derivatives (*e.g.* hydroxybenzoic acids, HBAs) and cinnamic acid derivatives (*e.g.* hydroxycinnamic acids, HCAs) (Khadem and Marles, 2010). Chlorogenic acids (CGAs) are ester compounds formed between *trans*-hydroxycinnamic acids and (-)-quinic acids [1L-1(OH), 3,4/5-tetrahydroxycyclohexane carboxylic acid] (Clifford et al., 2005; Jaiswal and Kuhnert, 2011; Jaiswal et al., 2014). The most studied CGAs are esters of quinic acids with coumaric-, caffeic- and ferulic acids, resulting in *p*-coumaroylquinic acids (*p*-CoQA), caffeoylquinic acids (CQA) and feruloylquinic acids (FQA) respectively (Jaiswal et al., 2014). In addition, CGAs occur in various structural forms due to multi-acylation on quinic acid that brings about different structural hierarchies namely *mono*-acyl, di-acyl, tri-acyl and, rarely, tetra-acyl derivatives (Jaiswal and Kuhnert, 2011). The CGA content varies from one plant to another. For instance, *tri*-CQA have been reported to have a limited presence in *Lactuca sativa* var. *Crispa* L. (Tamura et al., 2006), whilst artichoke has been reported to contain all mono-acyl and di-acyl CGAs (Sonnante et al., 2010).

CGAs are naturally-occurring plant defence metabolites that have been identified as resistance bio-markers, herbivore feeding retardants (Jansen et al., 2008) and are also induced when plants undergo bacterial and fungal infection (Marques and Farah, 2009). Previously we proposed a role for the interconversion of the phytoalexin - and phytoanticipin - roles of CGAs through storage and conjugation (Mhlongo et al., 2014). High contents of CGAs have been reported in plants such as pears, apples, arnica, artichoke, coffee beans, tobacco, burdock,

* Corresponding author.
** Corresponding author.
*E-mail addresses:* emadala@uj.ac.za (N.E. Madala), idubery@uj.ac.za (I.A. Dubery).

https://doi.org/10.1016/j.plaphy.2018.04.005
Received 20 February 2018; Received in revised form 3 April 2018; Accepted 3 April 2018
Available online 05 April 2018
0981-9428/ 

sweet potato, tomato, potato and eggplant amongst others (Niggeweg et al., 2004; Clifford et al., 2005; Docimo et al., 2016). CGAs are the main dietary bioactive phenolic acids and have been described to possess both nutritional and pharmacological properties (Ma et al., 2007; Falleh et al., 2008; Jaiswal et al., 2014; Hwang et al., 2014; Makola et al., 2016). A broad range of direct and indirect pharmacological properties has drawn interest as far as potential health benefits are concerned (Clifford et al., 2017).

The CGA compounds are biosynthesized *via* three routes in the phenylpropanoid pathway (Comino et al., 2009). The first route involves synthesis through the *trans*-esterification of the caffeoyl-CoA and quinic acid achieved by the hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT) enzyme, EC 2.3.1.99 (Comino et al., 2009; Sonnante et al., 2010). The second route implicates hydroxylation of *p*-coumaroyl quinate to CGAs by *p*-coumaroyl ester 3′-hydroxylase (C3′H, EC 1.14.13.36) (Comino et al., 2009; Sonnante et al., 2010), while the third involves hydroxylation of *p*-coumaroyl shikimic acid and caffeoyl shikimic acid which is further hydroxylated to caffeoyl-CoA. As such, the latter is a subsequent substrate for the hydroxycinnamoyl-CoA shikimate/quinate transferase (HCT, EC 2.3.1.133) enzyme (Comino et al., 2009; Sonnante et al., 2010).

The HCT enzymes from tobacco and tomato have high affinity for shikimate as substrate rather than quinate, and species that contain only this enzyme have been reported to have a low content of CGAs (Hoffmann et al., 2003). For instance, *Arabidopsis thaliana* contains both HCT and C3′H enzymes but no significant accumulation of CGAs (Niggeweg et al., 2004; Comino et al., 2009; Sonnante et al., 2010). Hoffmann et al. (2003) have indicated that silencing of the *hct* gene in tobacco results in accumulation of flavonoids but with no effect on the content of the CGAs (Hoffmann et al., 2003). Conversely, silencing of *hqt* greatly reduces the synthesis of CGAs in potato and redirected the metabolic flux to other phenylpropanoids (Niggeweg et al., 2004).

Phytochemical composition of globe artichoke (*Cynara cardunculus* var. *scolymus* L.), a member of *Asteraceae* family, has previously been studied extensively and shown to contain high levels of CGAs with the less common ones such as 1,3- and 1,5-*di*-caffeoylquinic acid as its major phenolics (Comino et al., 2009). Both HCT and HQT enzymes have previously been characterized and demonstrated to play an important role in the accumulation of CGAs in globe artichoke (Sonnante et al., 2010). High levels of *hqt* expression are correlated with a notable increase in biosynthesis of CGAs (Niggeweg et al., 2004; Sonnante et al., 2010; Chen et al., 2017). Furthermore, genetic transformation results have demonstrated that HQT is the key enzyme associated with CGA production in *Solanaceous* species (Niggeweg et al., 2004). More recently, the artichoke *hqt*1 gene was shown to enhance the production of CGAs and the synthesis of the artichoke metabolite, 1,5-*di*-CQA (cynarin) in transformed *Nicotiana* leaves (Sonnante et al., 2010).

Metabolomics, in particular non-targeted metabolomic approaches, has been widely adopted as a tool to investigate changes in the metabolism of genetically modified plants. Foremost, metabolomics offers a biochemical snapshot of an organism's phenotype (Tugizimana et al., 2014), thus it is only reasonable that this field has become an important complementary tool in investigating intended and unintended effects resulting from genetic modification. These include assessment of substantial equivalence (Catchpole et al., 2005; Kusano et al., 2011; Simó et al., 2014), evaluating the chemical diversity in transgenic tomatoes (Kusano et al., 2011), and comparing the amino acid composition in transgenic wheat (Baker et al., 2006). In addition, this approach has been applied in the evaluation of natural variation between GM soybean and non-GM seeds (Clarke et al., 2013). Herein, metabolomic approaches were applied to evaluate the effect of the artichoke *hqt*1 transgene on the composition of CGAs and related metabolites. Instead of the intended effect of increased levels of GCAs with a diversity resembling that of artichoke, metabolomic analysis uncovered evidence for silencing/inhibition of CGAs synthesis accompanied by an unintended rechanneling of metabolites towards BA derivatives.

## 2. Materials and methods

### 2.1. Tobacco cell lines for metabolomics studies

A stable transformant (hqt#7) overexpressing the globe artichoke (*Cynara cardunculus* subsp. *scolymus*) *hqt*1 gene (AM690438; Sonnante et al., 2010) was obtained through *Agrobacterium tumefaciens*-mediated transformation of *Nicotiana tabacum* (cv Samsun NN) under control of the 35S CaMV promoter. The transformant was confirmed to accumulate CGA (0.29 μg/mg FW as compared to 0.03 μg/mg FW in Samsun NN) in the T0 generation.

T2 transgenic plants were checked for *hqt*1 expression by semi-quantitative RT-PCR using artichoke *hqt*1 gene-vector specific primers. The PCR amplification experiments using synthesized cDNA as template with 10 μM of specific primers (below) were carried out using a One Taq® 2X Master Mix with standard buffer in 25 μL reaction volumes (Biolabs, Ipswich, Massachusetts, US). The thermal cycling program was as follow: one cycle of 94 °C for 3 min (initial denaturation), 30 cycles of 94 °C for 30 s (denaturation), annealing at 64 °C (*hqt1* gene primers) or 56 °C (*elf* 1α gene primers) for 1 min and 68 °C for 2 min (extension). Final extension was achieved at 72 °C for 10 min. Primer sequences were according to Sonnante et al. (2010): forward-*hqt1*: CCCAAGCTTATGGGGAGTGATCAGGCAAC; reverse-*hqt1*: GCTCTAGAC ATTACAACCAAAATCCTTA. The reference gene was *elongation factor1-α* from *N. tabacum,* D63396.1/|LOC107826390. The primer pair was designed using Integrated DNA Technologies primer quest tool (IDT, Coralville, IA, USA): forward-*elf*1α: CACACTGGAGGTTTTGAGG and reverse-*elf*1α: TGGAGTATTTGGGGGTGGT.

### 2.2. Preparation of cell suspension cultures and harvesting

For the transgenic cell line, callus was initiated from T2 transgenic *hqt*1 tobacco seeds germinated on solid Murashige and Skoog (MS) medium containing hormones ($0.25\ mg.L^{-1}$ of 2,4-dichlorophenoxyacetic acid and $0.25\ mg.L^{-1}$ of kinetin (pH 5.8)) and MS vitamins plus $100\ \mu g\ mL^{-1}$ kanamycin ($Kan^{100}$ as selectable marker) under sterile conditions. The kanamycin-resistant callus that formed from the germinating seedlings was then transferred to agar plates containing MS medium without kanamycin. Gene expression of *hqt*1 in the callus was verified by semi-quantitative RT-PCR as described above. Callus from the nontransformed Samsun NN cv was similarly obtained, but on kanamycin-free medium. Cell suspension cultures were established and cultivated at room temperature on a shaker at 130 rpm, with a light/dark cycle of 12 h/12 h, as described by Sanabria and Dubery (2006). After every 7 days, the cells were sub-cultured into fresh MS medium described above. Cells were harvested by centrifugation at 5100 × *g* for 15 min at 4°C, and the cell pellets were utilized for metabolite extraction. The experiment was carried out in five independent biological replicates for both transgenic and wild-type samples.

### 2.3. Extraction of metabolites

Metabolites were extracted by adding 100% methanol (in 1:1.5 m/v ratio), and homogenized using an Ultra-Turrax T8 homogenizer (IKA, Staufen, Germany) for 30 s. The homogenates were centrifuged at 5000 × *g* for 15 min using a benchtop swinging-bucket centrifuge at 4 °C. The supernatants were transferred into round bottom flasks, and the solvent evaporated to approximately 1 mL using a rotary evaporator at 55 °C. The 1 mL extracts were then further dried to completeness in a dry bath at 55 °C under constant air flow. The dried residues were reconstituted with 50% (v/v) aqueous LC-grade methanol (Romil Pure Chemistry, Cambridge, UK) and further filtered through 0.22-μm nylon filters into pre-labelled glass vials fitted with inserts and slitted caps. Samples were stored at −20 °C until analysis.