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Profiling of phenylpropanoids in transgenic low-sinapine oilseed rape (*Brassica napus*)

Karina Wolfram [a], Jürgen Schmidt [b], Victor Wray [c], Carsten Milkowski [a,1], Willibald Schliemann [a], Dieter Strack [a,*]

[a] Department of Secondary Metabolism, Leibniz Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle (Saale), Germany
[b] Department of Bioorganic Chemistry, Leibniz Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle (Saale), Germany
[c] Department of Structural Biology, Helmholtz Centre for Infection Research, Inhoffenstraße 7, D-38124 Braunschweig, Germany

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ABSTRACT

A dsRNA*i* approach silencing a key enzyme of sinapate ester biosynthesis (UDP-glucose:sinapate glucosyltransferase, encoded by the *UGT84A9* gene) in oilseed rape (*Brassica napus*) seeds was performed to reduce the anti-nutritive properties of the seeds by lowering the content of the major seed component sinapine (sinapoylcholine) and various minor sinapate esters. The transgenic seeds have been produced so far to the T6 generation and revealed a steady suppression of sinapate ester accumulation. HPLC analysis of the wild-type and transgenic seeds revealed, as in the previous generations, marked alterations of the sinapate ester pattern of the transformed seeds. Besides strong reduction of the amount of the known sinapate esters, HPLC analysis revealed unexpectedly the appearance of several minor hitherto unknown rapeseed constituents. These compounds were isolated and identified by mass spectrometric and NMR spectroscopic analyses. Structures of 11 components were elucidated to be 4-*O*-glucosides of syringate, caffeyl alcohol and its 7,8-dihydro derivative as well as of sinapate and sinapine, along with sinapoylated kaempferol glycosides, a hexoside of a cyclic spermidine alkaloid and a sinapine derivative with an ether-bridge to a C_6–C_3-unit. These results indicate a strong impact of the transgenic approach on the metabolic network of phenylpropanoids in *B. napus* seeds. Silencing of *UGT84A9* gene expression disrupt the metabolic flow through sinapoylglucose and alters the amounts and nature of the phenylpropanoid endproducts.

1. Introduction

Oilseed rape (*Brassica napus*) is one of the most important oilseed crops in temperate regions of the world. It accounts for up to 15% of the global oilseed production (Orlovius, 2003). The seeds contain approximately 50% oil and 25% proteins (w/w). After oil extraction, the resulting seed meal consists of approximately 40% proteins (Fenwick, 1982). Although *B. napus* is mainly an oilseed crop, its meal is rich in essential amino acids and therefore desirable for animal feeds and potential human food supplements (Ohlson, 1978; Shahidi and Naczk, 1992; Bell, 1993). However, the seeds accumulate large amounts of phenolic compounds, mainly sinapine and various other sinapate esters. The latter are predominantly derived from glucose and gentiobiose as well as from kaempferol glycosides (Baumert et al., 2005).

The major seed phenolic in *B. napus* is sinapine, which accumulates in the range 1–2% (w/w) of seed mass (Bell, 1993). Sinapate esters are considered as anti-nutritional factors as they confer astringency to the seed meal, lead to low digestibility of the seed proteins and cause a bitter taste (Fenwick, 1982; Kozlowska et al., 1990; Shahidi and Naczk, 1992; Naczk et al., 1998). Furthermore, phenolic compounds may form insoluble complexes with rapeseed proteins (Shahidi and Naczk, 1992). Hence, a main requirement for an efficient use of rapeseed as a protein crop is a substantial reduction of seed sinapate esters, especially sinapine. This prompted investigations of the genes involved in sinapine biosynthesis of *B. napus* to define targets for molecular breeding approaches to reduce the sinapate ester content. During the characterization of sinapate ester metabolism in *B. napus,* UGT84A9 (UDP-glucose:sinapate glucosyltransferase; EC 2.4.1.120) was identified as the key enzyme in sinapine biosynthesis (Milkowski et al., 2004; Mittasch et al., 2007). UGT84A9 catalyzes the transfer of glucose from UDP-glucose to sinapate forming 1-*O*-(*E*)-sinapoyl-β-glucose, which is the energy-rich acyl donor in reactions leading to sinapine and other sinapate esters in seeds and sinapoylmalate in seedlings (Baumert et al., 2005). Sinapoylmalate accumulates in

* Corresponding author. Tel.: +49 345 5582 1500; fax: +49 345 5582 1509.
E-mail address: dieter.strack@ipb-halle.de (D. Strack).
[1] Present address: Friedrich Schiller University Jena, Institute of General Botany, Am Planetarium 1, D-07743 Jena, Germany.

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leaves and plays an important role in UV-protection (Sheahan, 1996; Landry et al., 1995). Sinapine is assumed to contribute to the supply of choline for phosphatidylcholine in the young seedlings of *Raphanus sativus* (Strack, 1981). To reduce the sinapate esters in the seeds, a dsRNA*i* vector was used that silenced *UGT84A9* gene expression seed-specifically (Hüsken et al., 2005). The transgenic seeds of *B. napus* show a significant decrease in sinapine and various other sinapate esters. The best performing lines showed a reduction of total sinapate ester content to 24% for T3 seeds, compared to the corresponding wild-type seeds (Hüsken et al., 2005).

In continuation of our previous studies (Baumert et al., 2005; Hüsken et al., 2005), T6 seed extracts of spring oilseed rape (B*rassica napus* L. var. *napus* cv. Drakkar), suppressing *UGT84A9* gene expression (*UGT84A9*i) were analyzed by HPLC and compared to wild-type seeds. In addition to sinapine and total sinapate esters, the amount of 17 known phenolic compounds, mainly hydroxycinnamate esters, were determined. Surprisingly, the reduction of the sinapate ester content in *UGT84A9*i seeds was accompanied by an increase of various hitherto unknown phenolic compounds, which, however, could not compensate for the net loss in the total sinapate ester content. These compounds were isolated and identified by a combination of high-field NMR spectroscopy and high resolution electrospray ionization mass spectrometry. Detailed qualitative and quantitative characterization of the altered pattern of phenolic compounds in the transgenic seeds is a necessary prerequisite to reveal substantial equivalence (FAO/WHO, 2000).

2. Results and discussion

2.1. Alterations of the pattern of secondary metabolites in transgenic rapeseed

To achieve suppression of biosynthesis of sinapine (Fig. 1) and related sinapate esters (Baumert et al., 2005), silencing of *UGT84A9* gene expression was carried out. *B. napus* was transformed with the plasmid pLH–UGT84A9–GUS under the control of the seed-specific napin promoter (Hüsken et al., 2005). This dsRNA*i* construct contains a part of the *UGT84A9*-encoding region as an inverted repeat. For T3 seeds (from a homozygous T2 plant) it could be shown, that the best line afforded a reduction of total sinapate ester content to 24% compared to wild-type seeds. Furthermore the reduction of sinapine was – at least up to the transgenic T3 generation – a stable trait that does not appear to interfere with other important seed characteristics like oil, protein or glucosinolate and eruic acid contents.

The transgenic plants have been propagated so far to the T6 generation and seeds were analyzed for the steady suppression of sinapate ester accumulation. The average reduction of the content of sinapine and total sinapate esters of wild-type and *UGT84A9*i seeds were determined. For this purpose, five replicates of seeds (20 mg each) were extracted with aqueous methanol and analyzed by HPLC. The results are summarized in Fig. 2. An average reduction of the sinapine content to 40% and total sinapate ester content to 36% for T6 seeds were observed, compared to wild-type seeds. It reveals a steady suppression of the sinapate ester accumulation through the preceding rapeseed generations.

In Fig. 3 the HPLC elution profiles of extracts from wild-type (A) and *UGT84A9*i (B) seeds are shown. For *UGT84A9*i seeds, the content of many sinapate esters such as 1-*O*-(*E*)-sinapoyl-β-glucose, 2-*O*-(*E*)-sinapoyl-*S*-malate and 1,2-di-*O*-(*E*)-sinapoyl-β-glucose are below the HPLC detection limit. Table 1 summarizes a semi-quantitative comparison of the seed constituents **A**–**Q** for wild-type and *UGT84A9*i seeds. These results substantiate the pivotal role of 1-*O*-sinapoyl-β-glucose for the formation of the complex

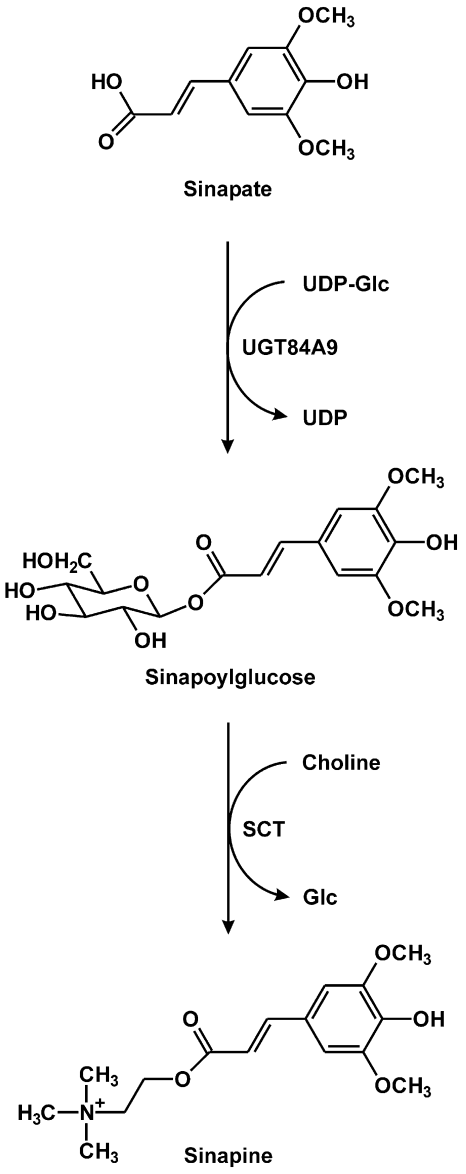

Fig. 1. Formation of sinapine via sinapoylglucose in seeds of the Brassicaceae, showing the cruical role of UGT84A9 (UDP-glucose:sinapate glucosyltransferase, EC 2.4.2.120) in this pathway. Glc = Glucose, SCT = (1-*O*-sinapoylglucose:choline sinapoyltransferase, E 2.3.1.91).

pattern of sinapate esters in *B. napus* seeds and is in agreement with a previous study (Baumert et al., 2005).

The reduction of the amounts of sinapine and other sinapate esters in *UGT84A9*i seeds was accompanied by an increase of several minor phenolic compounds (**1**–**11** in Fig. 3B), which were below the HPLC detection limit in wild-type seeds. In a related study (K. Wolfram, unpublished) using LC-MS, these compounds were also found in the control seeds in trace amounts. The structure analysis of these components by mass spectrometry and NMR spectroscopy revealed the presence of several 4-*O*-glucosides of syringate, caffeyl alcohol, sinapate and sinapine. Furthermore, sinapoylated kaempferol glycosides, a hexoside of a cyclic spermidine alkaloid and a sinapine derivative bridged to a C_6–C_3-unit were detected. The presence of these compounds in *UGT84A9*i seeds indicate a strong impact of the transgenic approach on the phenylpropanoid network, not only leading to the reduction of the known sinapate esters but also to an unpredictable increase of several 4-*O*-glucosides. It is tempting to speculate that in these