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BIOSYNTHESIS OF GLUCOSINOLATES IN THE DEVELOPING SILIQUE WALLS AND SEEDS OF *SINAPIS ALBA*

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Key Word Index—*Sinapis alba*; Brassicaceae; developing silique; glucosinolates; biosynthesis.

Abstract—When [^{14}C]tyrosine was administered to developing seeds and silique walls of *Sinapis alba* L. both tissues *de novo* synthesized *p*-hydroxybenzylglucosinolate (*p*-OHBG). Within a silique, the incorporation rate of tyrosine to *p*-OHBG was up to 70-fold higher in the silique walls compared to the seeds as evidenced by both *in vivo* and *in vitro* measurements. During development, the amount of *p*-OHBG increases in the seeds, whereas the level remains relatively constant in the corresponding walls in radioisotope feeding experiments using intact siliques. This shows that *p*-OHBG is translocated from the walls to the seeds in *S. alba*. Although translocation is a major contributor to glucosinolate accumulation in the seeds, we also demonstrate that seeds of *S. alba* contain all the enzymes required for *de novo* biosynthesis of *p*-OHBG. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Glucosinolates are sulphur- and nitrogen-containing glucosides derived from amino acids [1]. They are found in all members of the Cruciferae, which includes the agriculturally important oilseed rape, *Brassica napus* L. The degradation products of glucosinolates are potentially toxic to animals, which feed on the oilseed meal-cakes [2]. Although traditional breeding has reduced the content of glucosinolates in rape seeds, the concentration of glucosinolates in a given variety fluctuates as a response to growth conditions and seasonal variations [3]. Tissue-specific elimination of glucosinolates is desirable and may be achieved using molecular techniques.

The floral tissue is the major site for accumulation of glucosinolates in oilseed rape [4]. The amount of glucosinolates produced in the vegetative tissues of oilseed rape represents a small fraction of the amount of glucosinolates, which accumulate in the floral tissue, and is therefore unlikely to contribute significantly to the accumulation of glucosinolates in the seed [4]. Grafting experiments by Lein [5] have indicated that the siliques are the main site for biosynthesis of those glucosinolates which are stored in the seed. Studies with oilseed rape have indicated that the wall of the silique is the major site for biosynthesis of the glucosinolates found in the seeds [6–10]. This implies that the glucosinolates synthesized in the silique wall are subsequently translocated to the seed. Toroser *et al.* [11] have shown that a continuous increase in the accumulation of ^{35}S-labelled glucosinolates in the seed is correlated with a progressive decrease in labelled glucosinolates in the silique wall. Zhao *et al.* [10] have demonstrated that allylglucosinolate exogenously added to intact siliques is rapidly translocated from the wall to the seeds. Gijzen *et al.* [12] have shown that excised, immature rapeseed embryos take up exogenously supplied glucosinolates by a carrier-mediated transport system. The synthesis of glucosinolates in the walls and the subsequent translocation of glucosinolates from the wall to the seeds have led to the assumption that none of the enzymes in glucosinolate biosynthesis is present in the seeds. This assumption is supported by the observation by Bergmann [6] that [^{35}S]sulphate was only incorporated into the seeds in the presence of silique walls of *Sinapis alba* L., and by the report by Gijzen *et al.* [13] that administration of desulphoglucosinolates to excised embryos from rape seeds harvested 24 days after pollination did not result in an increase in the content of glucosinolates in the embryos. Recently, however, Toroser *et al.* [11, 14] have reported that isolated rape seeds harvested 21 days after pollination were able to convert desulphoglucosinolate precursors into glucosinolates. This provides evidence for the occurrence of a PAPS-sulphate transferase in seeds. It has not

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*Author to whom correspondence should be addressed. Tel, 00 45 35 28 33 54; Fax, 00 45 35 28 33 33; E-mail, halkier@biobase.dk been demonstrated unequivocally whether seeds carry out other reactions in the biosynthetic pathway of glucosinolates.

We have chosen *Sinapis alba*, as a model plant for studies on the biosynthetic capacity of glucosinolate-containing seeds. *S. alba* contains only one major glucosinolate, *p*-hydroxybenzylglucosinolate (*p*-OHBG), which is derived from tyrosine. In contrast, the major glucosinolates in oilseed rape are synthesized from chain-elongated amino acids which are not commercially available as radioactively labelled precursors. In this report, we describe the first experimental evidence that seeds of *S. alba* contain the enzymes necessary for biosynthesis of *p*-OHBG. Our results show that some of the glucosinolates in the seeds are synthesized in the seeds.

RESULTS AND DISCUSSION

De novo *biosynthesis of glucosinolates in seeds and silique walls harvested* 2 *to* 5 *weeks after pollination*

Isolated seeds and silique walls of *S. alba* incubated with [^{14}C]tyrosine for 24 h produced a radioactively labelled compound comigrating with authentic *p*-OHBG when analyzed by TLC (Fig. 1A) and by HPLC (data not shown). The ability of isolated seeds to convert tyrosine to *p*-OHBG demonstrates that seeds contain all the enzymes in the biosynthetic pathway of *p*-OHBG. The biosynthetic activity increased in the developing seed in the first weeks after pollination (Fig. 1A). In a typical experiment, seeds harvested 2 weeks after pollination produced approx. 0.5 nCi *p*-OHBG/seed when incubated 24 h with trace amounts of [^{14}C]tyrosine, whereas seeds harvested 5 weeks after pollination produced approx. 8 nCi *p*-OHBG/seed (Fig. 1A). In the same experiment, approx. 35 nCi *p*-OHBG/wall were incorporated into the isolated silique walls independent of developmental stage (Fig. 1B). The incorporation rate in the walls was up to 70 times higher compared to the activity in the corresponding seeds, which indicates that in *S. alba* the silique wall is the major site for glucosinolates biosynthesis, as has also been indicated in oilseed rape [6–10].

In a typical experiment, the content of unlabelled *p*-OHBG in the largest seed in the silique increased approximately 6-fold during development of the siliques from 2 to 5 weeks after pollination (Fig. 2). The content of *p*-OHBG in the corresponding walls remained relatively constant (Fig. 2). The majority of *p*-OHBG which accumulate in the seed is translocated from the walls as demonstrated by a steady accumulation of *p*-OHBG in the developing seed and a low *de novo* biosynthesis of *p*-OHBG in the seed (Fig. 2). Similarly, the relatively constant level of *p*-OHBG in the walls combined with the high biosynthetic activity in the walls shows that *p*-OHBG is translocated away from the walls.

Toroser *et al.* [11, 14] have shown that a PAPS-sulphate transferase is present in seeds of oilseed rape. In the same study, the inability of isolated seeds of

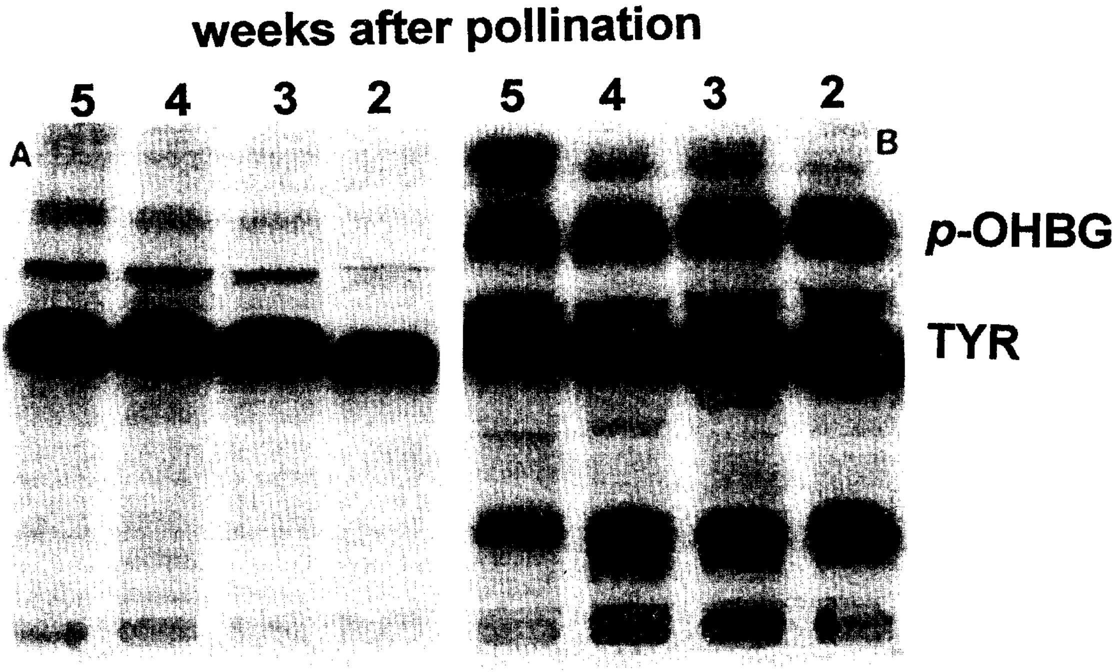

Fig. 1. *De novo* biosynthesis of *p*-OHBG from tyrosine by seeds and silique walls of *S. alba* harvested 2, 3, 4 and 5 weeks after pollination. Isolated seeds (A) and silique walls (B) were fed with 0.1 μCi [^{14}C]tyrosine for 24 h and extracted with 90% methanol. Aliquots of the extracts were analyzed by TLC. The radioactive bands were visualized using a phosphor imager. Abbreviations—TYR, tyrosine; *p*-OHBG, *p*-hydroxybenzylglucosinolate.

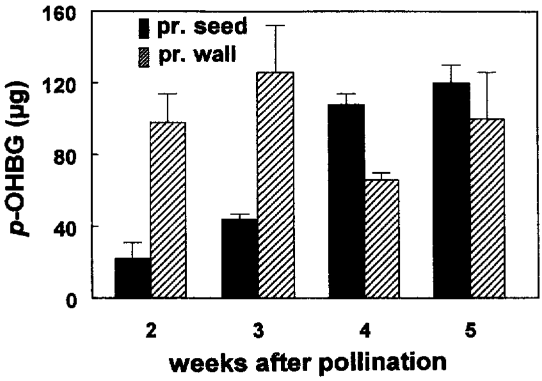

Fig. 2. The content of *p*-OHBG in the wall and largest seed of siliques harvested 2, 3, 4 and 5 weeks after pollination. The siliques were dissected into seeds and walls followed by extraction in 90% methanol. The content of *p*-OHBG was quantified by HPLC as described in "Experimental". Abbreviations—*p*-OHBG, *p*-hydroxybenzylglucosinolate.

oilseed rape to incorporate ^{35}S-labelled sulphate into the thiol moiety of glucosinolate within a 3 h incubation period made the authors suggest that seeds of oilseed rape cannot synthesize desulphoglucosinolates from amino acids [11]. An alternative explanation is that the administered ^{35}S-sulphate was not converted into the immediate sulphur donor of the thiol moiety of glucosinolates. Based on the demonstration of *de novo* biosynthetic activity of *p*-OHBG in seeds of *S. alba* we consider it likely that seeds of the taxonomically related oilseed rape also synthesize glucosinolates.

Comparison of in vitro *biosynthetic activity in microsomes from different tissues of* S. alba

Microsomes were prepared from seeds, walls, and green leaves, and from etiolated seedlings of *S. alba* and their *in vitro* biosynthetic activity was measured by the conversion of trace amounts of radioactively labelled tyrosine to oxime. Microsomes prepared from green leaves were most active (Fig. 3). Microsomes from jasmonic acid-induced etiolated seedlings and from isolated walls had activities corresponding to 30% and 6% of the activity in the green leaves. No activity was detected in microsomes isolated from seeds. This supports the *in vivo* data, that seeds only have limited biosynthetic activity compared with the silique wall. The high *in vitro* biosynthetic activity in the green leaves of *S. alba* compared with the other tissues suggests that the leaves might contribute to the accumulation of *p*-OHBG in the siliques.

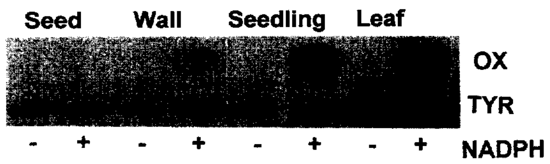

Fig. 3. Comparison of *in vitro* biosynthetic activity in microsomes isolated from different tissues of *S. alba*. Microsomes prepared from seeds, silique walls, and green leaves, and from etiolated seedlings treated with 50 µM jasmonic acid dissolved in 50% ethanol were incubated with 1 µCi [^{14}C]tyrosine in the presence or absence of NADPH for 30 min at 35°. The reactions were stopped by ethyl acetate extraction. The extracts were analyzed by TLC. Radioactive bands were visualized and quantified using a phosphor imager. Abbreviations—TYR, tyrosine; OX, *p*-hydroxyphenylacetaldoxime.

Distribution of [^{14}C]p-*OHBG between seeds and walls after feeding* [^{14}*C*]*tyrosine to intact siliques*

Intact siliques harvested 5 weeks after pollination were incubated with ^{14}C-labelled tyrosine. After incubation, the siliques were immediately dissected into seeds and silique walls for analysis of the distribution of *de novo* synthesized ^{14}C-labelled *p*-OHBG. Increasing amounts of ^{14}C-labelled *p*-OHBG accumulated in extracts of walls with incubation time (Fig. 4A). A similar pattern was observed in extracts from seeds, except that the accumulation of labelled *p*-OHBG in seeds was delayed a few hours compared to walls (Fig. 4B). Low amounts of [^{14}C]tyrosine accumulated in the seeds (Fig. 4B). This observation combined with the low *in vitro* biosynthetic acticity measured in seeds (Fig. 3), indicate that the majority of the *p*-OHBG which accumulates in the seeds is translocated from the silique walls.

Toroser *et al.* [14] have shown that the individual glucosinolates in oilseed rape are differently partitioned between seeds and walls. A partition quotient, P*q*, was defined as an arbitrary measure of the ratio of newly synthesized glucosinolate in seeds and walls after incubation of siliques with [^{35}S]sulphate [14]. When calculated after 48 h of incubation, the major glucosinolates in oilseed rape (2-hydroxybut-3-enyl glucosinolate, but-3-enyl glucosinolate and 4-hydroxyindolyl-3-methyl glucosinolate) had high P*q* values between 3 and 7, whereas others were below 1 [14]. The different P*q* values measured for the individual glucosinolates in oilseed rape suggest that (1) a common transport system has different affinities for different glucosinolate side chains or (2) that each glucosinolate has its own transport system which operates at a different efficiency. We have measured a P*q* of 0.3 in *S. alba* after feeding with [^{14}C]tyrosine for 30 h. The P*q* values vary with time, being only 0.2 after 2 h. This might reflect that a certain level of glucosinolates has to build up in the wall before translocation to the seed takes place.

In conclusion, we have shown that the developing seeds of *S. alba* are capable of converting tyrosine to *p*-OHBG, which demonstrates that the seeds contain the enzymes required for *de novo* biosynthesis of glucosinolates. Additionally, we have shown that the silique wall is the major site for glucosinolate biosynthesis in *S. alba* and that glucosinolates subsequently is translocated to the seed. It has been hypothesized

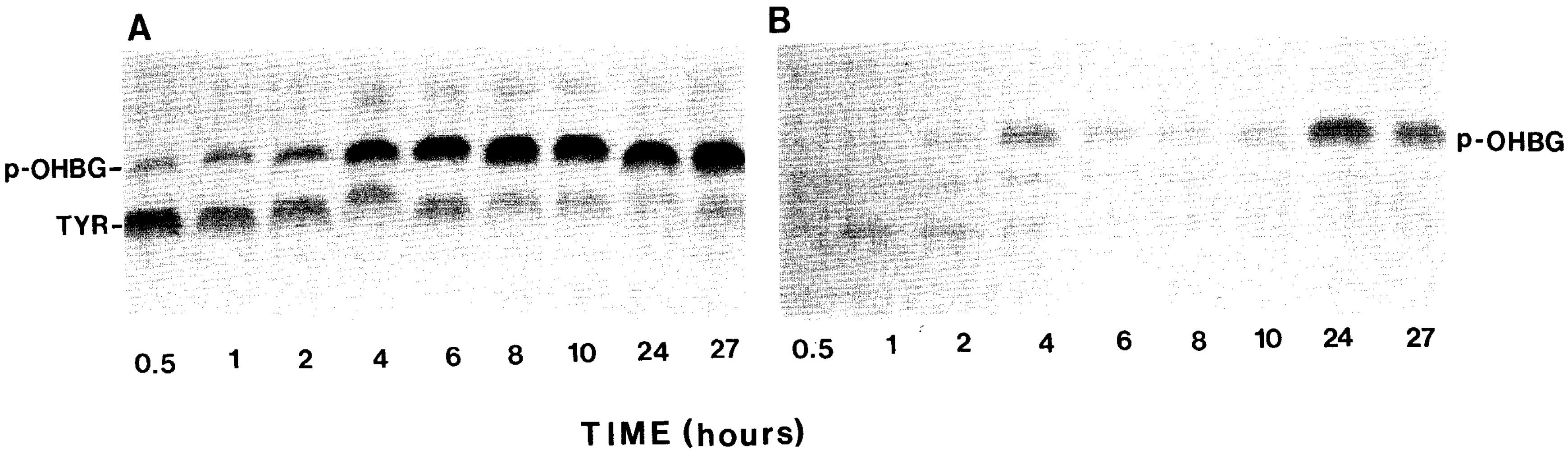

Fig. 4. Distribution of [^{14}C]*p*-OHBG between seeds and walls after feeding [^{14}C]tyrosine to intact siliques. Siliques were harvested 5 weeks after pollination and incubated with 0.1 μCi [^{14}C]tyrosine. After incubation, the siliques were dissected into walls (A) and seeds (B) and *p*-OHBG was extracted. The extracts were analyzed by TLC and the radioactively labelled *p*-OHBG was visualized by autoradiography. Abbreviations—TYR, tyrosine; *p*-OHBG, *p*-hydroxybenzylglucosinolate.

that glucosinolate-free oilseeds could be produced by disruption or by down-regulation of the glucosinolate transport system. Our results show that blockage of the transport of glucosinolates from walls to seeds will not result in the production of glucosinolate-free seeds.

EXPERIMENTAL

Materials

Seeds of *Sinapis alba* L. were obtained from Prodana Seeds A/S, Odense, Denmark. All plants were grown in a greenhouse at 15–18° with 16 h light and 8 h darkness. Intact siliques were harvested 2 to 5 weeks after pollination. L-[U-^{14}C]Tyrosine (443 mCi mmol^{-1}) was purchased from Amersham. *p*-[U-^{14}C]hydroxyphenylacetaldoxime (394 mCi mmol^{-1}) was enzymatically synthesized from L-[U-^{14}C]tyrosine as described previously [15]. *p*-Hydroxybenzylglucosinolate was a generous gift from Professor Anders Kjær.

Administration of [^{14}C]*tyrosine and* [^{14}C]*oxime to seeds and walls of siliques harvested at* 2 *to* 5 *weeks after pollination*

Siliques from *S. alba* were harvested 2, 3, 4 and 5 weeks after pollination and dissected into seeds and silique walls by cutting along their dehiscence zones. A seed (represented by the largest seed in the silique) and the wall of the harvested siliques were incubated with 0.1 μCi [^{14}C]tyrosine or [^{14}C]oxime tracer for 24 h at 22° under continuous light (60 μmol photon m^{-2} s^{-1}, 400–700 nm light provided by fluorescent lights). H_2O was added to the test tubes as needed to prevent the tissues from drying out. After incubation, seeds and walls were rinsed with H_2O, blotted on to Whatman 3M filter paper, followed by extraction of glucosinolates. All experiments were carried out in duplicate.

Measurement of in vitro *biosynthetic activity in microsomes from different tissues of* S. alba

Microsomes were prepared from green leaves, seeds, and silique walls of plants 5 weeks after pollination, and from one week old, etiolated seedlings treated with 50 μM jasmonic acid in 50% ethanol as described earlier [16]. The microsomal preparations (1 mg protein) were included in reaction mixtures which contained 1 μCi of [^{14}C]tyrosine (443 mCi mmol^{-1}), and 2 mM NADPH in a total volume of 280 μl of 50 mM Tricine, pH 7.9, 2 mM DTT. The reactions were started by addition of NADPH. After 30 min incubation at 35°, the reactions were stopped by extraction with ethyl acetate. The ethyl acetate extracts were concentrated and applied to TLC plates eluted in a solvent system of toluene–ethyl acetate (5:1). Production of radioactively labelled oxime was visualized and quantified using a phosphor imager (Storm 840, Molecular Dynamics).

Time-course for administration of [^{14}C]*tyrosine to intact siliques*

Siliques were harvested 5 weeks after pollination and 0.1 μCi [^{14}C]tyrosine was administered to the intact siliques through the petiole via the transpiration stream. H_2O was added to the tubes as needed to prevent the tissue from drying out. After incubation for given time periods, the intact siliques were rinsed with H_2O and blotted on to Whatman 3M filter paper. The siliques were dissected immediately into seed and wall fractions by cutting along their dehiscence zones followed by extraction of glucosinolates. All experiments were carried out in duplicate.

Extraction and analysis of p-*OHBG*

Glucosinolates were extracted from the plant materials with 90% methanol as previously described [16]. The extracts were dried *in vacuo*, redissolved in 50% methanol, and analyzed using TLC eluted in 2-propanol–ethyl acetate–H_2O (7:1:2) as described previously [17]. The radioactively labelled *p*-OHBG was visualized by autoradiography or by a phosphor imager. The total content of *p*-OHBG was quantified by HPLC equipped with a UV monitor as described previously [16]. An aliquot of extracts was applied to a Nucleosil 100-10C$_{18}$ column (250 mm × 4.6 mm) and eluted isocratically with 1% solvent B (70% methanol) in solvent A (0.1 M ammonium acetate) at a flow rate of 1 ml/min. The content of unlabelled *p*-OHBG was calculated by integration of the peak areas corresponding to *p*-OHBG on the elution profile obtained by continuous monitoring of UV absorption.

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