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Studies on the late steps of (+) pisatin biosynthesis: Evidence for (−) enantiomeric intermediates

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Abstract

Pisatin, a 6a-hydroxyl-pterocarpan phytoalexin from pea (*Pisum sativum* L.), is relatively unique among naturally occurring pterocarpans by virtue of the (+) stereochemistry of its 6a–11a C–C bond. However, pisatin synthesizing pea tissue has an isoflavone reductase, first identified in alfalfa, which acts on the (−) antipode. In order to establish the natural biosynthetic pathway to (+) pisatin, and to evaluate the possible involvement of intermediates with a (−) chirality in its biosynthesis, we administered chiral, tritium-labeled, isoflavanones and pterocarpans to pisatin-synthesizing pea cotyledons and compared the efficiency of their incorporation. Pea incorporated the isoflavanone, (−) sophorol, more efficiently than either its (+) antipode, or the pterocarpans (+) or (−) maackiain. (−) Sophorol was also metabolized by protein extracts from pisatin-synthesizing pea seedlings in a NADPH-dependent manner. Three products were produced. One was the isoflavene (7,2′-dihydroxy-4′,5′-methylenedioxyisoflav-3-ene), and another had properties consistent with the isoflavanol (7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol), the expected product for an isoflavanone reductase. A cDNA encoding sophorol reductase was also isolated from a cDNA library made from pisatin-synthesizing pea. The cloned recombinant sophorol reductase preferred (−) sophorol over (+) sophorol as a substrate and produced 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol. Although no other intermediates in (+) pisatin biosynthesis were identified, the results lend additional support to the involvement of intermediates of (−) chirality in (+) pisatin synthesis.

Keywords: *Pisum sativum* L.; Leguminosae; Pea; Biosynthesis; Isoflavonoids; Pterocarpans

1. Introduction

The pterocarpan pisatin (**5**) (see Fig. 1) from garden pea was the first chemically identified phytoalexin (Cruickshank and Perrin, 1960) and is a product of the isoflavonoid pathway (Dixon, 1999). The basic pterocarpan carbon skeleton contains rings A–D as shown for pisatin (**5**) in Fig. 1A. In nature, pterocarpans exist in two stereoisomeric forms, one of which is exemplified by (+) pisatin (**5**) (Fig. 1A). Unlike pea, most legumes produce the (−) stereoisomer of pterocarpans (Dewick, 1988) and have the configuration at asymmetric carbons 6a and 11a shown for (−) maackiain (**3b**) (Fig. 1A). The late steps of pisatin (**5**) biosynthesis, particularly the steps that lead to the (+) enantiomer and to hydroxylation at the 6a carbon, are not understood. Most models for pterocarpan biosynthesis involve reduction of an isoflavone, catalyzed by isoflavone reductase (IFR), as an intermediate step in the biosynthesis of these compounds (Aoki et al., 2000; Dixon, 1999).

IFR was first detected in chickpea (Tiemann et al., 1987) and cDNAs encoding IFR have been cloned from chickpea, alfalfa, pea and *Lotus japonicus* (Paiva et al., 1991,

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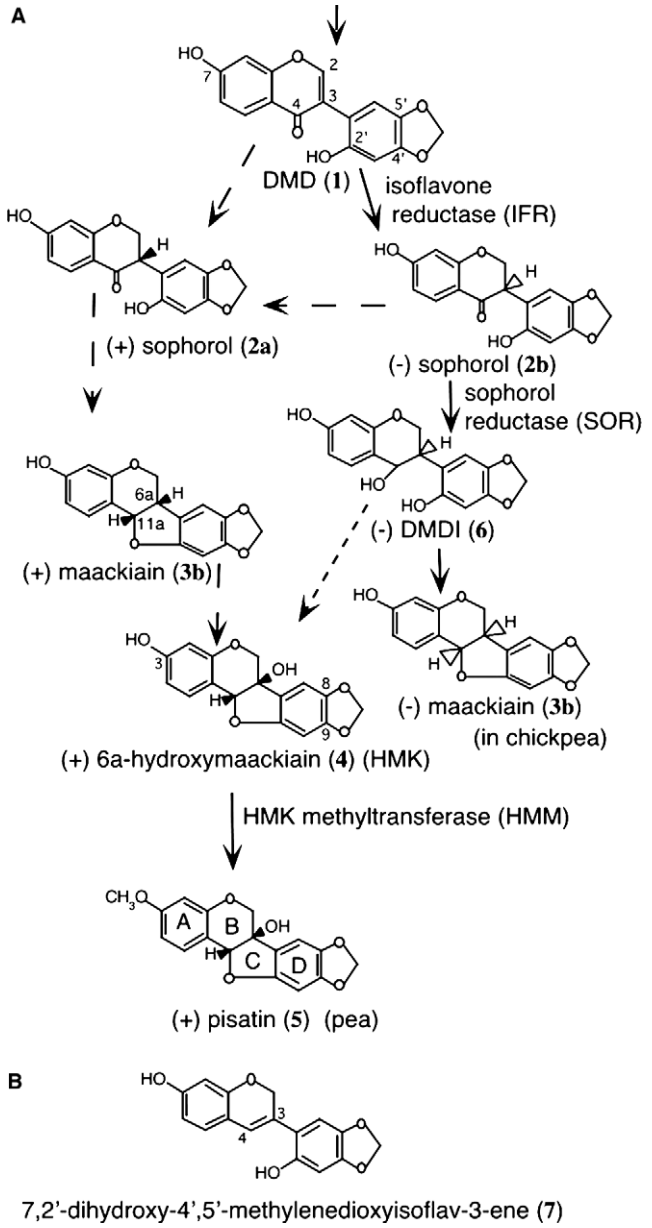

Fig. 1. (A) Proposed pathways for the biosynthesis of (+) pisatin (**5**) and (−) maackiain (**3b**). The pathway proposed by Banks and Dewick (1982) for (+) pisatin (**5**) is indicated by dashed arrows. Reactions for which the enzymatic activity has been confirmed are indicated by solid arrows. The occurrence of 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol (DMDI) (**6**) as an intermediate in the (+) pisatin (**5**) pathway is indicated by a dotted arrow. DMD = 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavone. (B) Structure of 7,2′-dihydroxy-4′,5′-methylenedioxyisoflav-3-ene (**7**).

1994; Shimada et al., 2000; Tiemann et al., 1991). IFR is of special significance, as the reduction of the 2,3 double bond is the first reaction in the pterocarpanoid pathway that produces an asymmetric product. Dewick and Ward (1977) proposed that this reaction could be the branchpoint leading to the two pterocarpan stereoisomers and that the IFR leading to (−) pterocarpans, converts optically inactive isoflavones to (−) isoflavanones and the IFR leading to (+) pterocarpans does the converse.

The IFR that has been isolated and characterized from alfalfa, a plant which makes only (−) pterocarpans, only converts optically inactive isoflavones into (−) isoflavanones (Paiva et al., 1991), supporting the prediction of Dewick and Ward that there is a specific reductase for the synthesis of (−) pterocarpans. However, the IFR enzymatic activity detected in pea tissue that synthesizes (+) pisatin (**5**) and the IFR encoded by an *Ifr* cDNA isolated from this tissue, catalyze the conversion of the achiral isoflavone 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavone (DMD) (**1**) to the (−) isoflavanone (−) "sophorol" (**2b**) (Fig. 1A) (Paiva et al., 1994). In alfalfa, the final two enzymes that form (−) medicarpin (3-hydroxy-9-methoxy pterocarpan) from the (−) isoflavanone (vestitone: 7,2′-dihydroxy-4′-methoxyisoflavanone) are isoflavanone reductase (vestitone reductase, VR), which acts on (−) vestitone to make (−) 7,2′-dihydroxy-4′-methoxyisoflavanol (DMI), and the isoflavanol dehydratase that dehydrates (−) DMI to make (−) medicarpin (Guo et al., 1994a,b) (structures not shown). A cDNA encoding VR from alfalfa has been cloned (Guo and Paiva, 1995).

Another uncertainty in the biosynthesis of (+) pisatin (**5**) involves the origin of the oxygen in the hydroxyl group at 6a. Radiolabeling studies in pea found that (+) maackiain (**3a**) is readily incorporated into (+) pisatin (**5**). ^{2}H NMR analysis of pisatin (**5**), which had incorporated (+) [11a-^{2}H, 6-^{2}H] maackiain (**3a/b**), revealed that both labeled protons were retained, suggesting that hydroxylation at the 6a carbon probably involved an O_2-dependent oxygenase and did not desaturate either the 11a–6a or 6–6a carbon bonds (Banks and Dewick, 1983a,b). However, subsequent labeling experiments with $^{18}O_2$ and $H_2{}^{18}O$ failed to support this model for the origin of the 6a-hydroxyl group in pisatin (**5**) and indicated that direct hydroxylation of maackiain (**3**) is not involved in (+) pisatin (**5**) biosynthesis (Matthews et al., 1987, 1989).

While the step(s) responsible for determining the stereochemistry of (+) pisatin (**5**) have yet to be described, all of the data indicate that the final step in pisatin (**5**) biosynthesis is methylation of (+) 6a-hydroxymaackiain (**4**) at the 3-0 position (Fig. 1A). A methyl transferase, (+) 6a-hydroxymaackiain 3-*O*-methyltransferase (HMM), that catalyzes this reaction has been purified from pea (Preisig et al., 1989). cDNA clones encoding HMM have been isolated and used to make antisense (anti *Hmm*) constructs of *Hmm*. When anti *Hmm* constructs are expressed in transgenic pea tissue (hairy roots), the tissue has a decreased ability to produce pisatin (**5**) (Wu et al., 1997; Wu and VanEtten, 2004).

The purpose of this present study was to evaluate further the participation of the (−) and (+) isoflavonoid intermediates in the synthesis of (+) pisatin (**5**). This was done by measuring the relative incorporation of sophorol (**2**) and maackiain (**3**) enantiomers into (+) pisatin (**5**) and by determining if an enzyme (sophorol reductase) and a gene analogous to (−) *Vr* from alfalfa are present in pea.

2. Results and discussion

2.1. In vivo labeling

Most previous studies of the incorporation of radiolabel from possible precursors into pisatin (**5**) have used immature pea pods (e.g., Banks and Dewick, 1983a,b). We developed a method, which uses cotyledons from 4- to 5-day old pea seedlings and allows quick production of pea tissue for such studies. The cotyledons synthesize pisatin (**5**) upon wounding and the amount produced is increased if $CuCl_2$ is applied to the wounded tissue (Fig. 2). When tritiated (−) sophorol (**2b**) was administered to such tissue, it was rapidly incorporated into pisatin (**5**) and by 8 h little of the added sophorol (**2**) was detectable (Fig. 3).

Tritiated (+) and (−) sophorol (**2a**) and (**b**) and (+) and (−) maackiain (**3a**) and (**b**) were individually administered to wounded cotyledons, which had been treated with 5 mM $CuCl_2$. The (+) pisatin (**5**) synthesized during the incubation period was purified and the amount of tritium incorporated into it measured (Table 1). The time which elapsed between addition of $CuCl_2$ and the tritiated isoflavonoids and the length of precursor administration were varied to give six different conditions. (−) Sophorol (**2b**) was incorporated better than any of these other compounds in all six conditions except for two individual treatments in which there was no significant difference: (+) sophorol (**2a**) in experiment A and (+) maackiain (**3a**) in experiment F (Table 1). The incorporation of tritiated DMD into (+) pisatin (**5**) was assayed one time and was similar to that of (−) sophorol (**2b**) and both were better incorporated than any of the other compounds tested (Table 1).

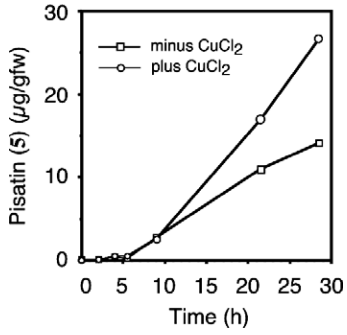

Fig. 2. Production of pisatin (**5**) by pea cotyledons. Cotyledon sections were prepared from 4-d old seedlings and the cut surfaces treated with 5 mM $CuCl_2$ (+ $CuCl_2$) or H_2O (− $CuCl_2$) as described in Section 4.

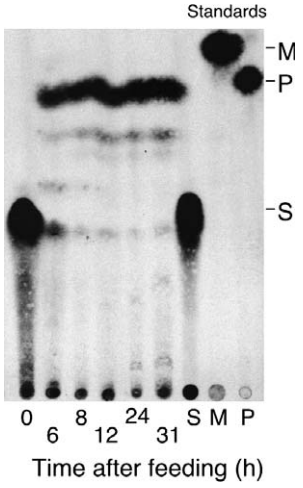

Fig. 3. In vivo incorporation of (−) sophorol (**2b**) into pisatin (**5**). Twenty-five nmol [^{3}H] (−) sophorol (4.0 Ci/mol), in 10 μl of 2% DMSO and 5 mM $CuCl_2$ were administered to each of two 6-day old cotyledon sections. Samples were collected and extracted into ethyl acetate as indicated in the text. The ethyl acetate soluble compounds were separated by TLC and the TLC plates subjected to autoradiography. The 3 lanes to the right contain standards: S = [^{3}H] sophorol (**2**), M = [^{3}H] maackiain (**3**) and P = [^{14}C] pisatin (**5**).

Though Banks and Dewick (1983b) had demonstrated that (+) maackiain (**3a**) is incorporated into (+) pisatin (**5**) in vivo, the current study is the first to compare the incorporation efficiencies of both isomers of sophorol (**2**) and maackiain (**3**) in the same experiment. The superior incorporation of (−) sophorol (**2b**) is consistent with a model of pisatin (**5**) biosynthesis that depends on an isoflavone reductase, which produces the (−) isoflavanone from DMD during the biosynthesis of pisatin (**5**) rather than the (+) enantiomer and is consistent with the lack of an isomerase that produces (+) sophorol (**2a**). As indicated previously, the *Ifr* isolated from pea has been shown to encode a protein that produces (−) sophorol (**2b**) from DMD (**1**) (Paiva et al., 1994). Recently, Wu and VanEtten (2004) have shown that pea hairy roots containing sense and antisense constructs of pea *Ifr* have a reduced ability to make pisatin (**5**), which is also consistent with a pisatin (**5**) biosynthetic pathway that involves (−) sophorol (**2b**) as an intermediate rather than (+) sophorol (**2a**) (Fig. 1A). In addition, the observed better incorporation of (−) sophorol (**2b**) compared to (+) maackiain (**3a**) would not be expected if (+) maackiain (**3a**) were a normal intermediate in the pathway at the position shown in Fig. 1A: (−) sophorol (**2b**) is placed upstream of (+) maackiain (**3a**) in models that propose the latter as an intermediate. As expected, the incorporation of (−) maackiain (**3b**) into (+) pisatin (**5**) was generally poor, consistent with its lack of involvement in any of the proposed pathways for (+) pisatin (**5**) biosynthesis.

2.2. Metabolism of (−) sophorol (2b) by pea proteins

Since the administration experiments suggested that (−) sophorol (**2b**) is an intermediate in (+) pisatin (**5**) biosynthesis, protein extracts of $CuCl_2$-treated pea seedlings were assayed for enzymatic activity with (−) sophorol (**2b**) as a substrate. When incubated with NADPH, three new compounds, one of which accumulated to a significant amount, were detected (Fig. 4). The major product (“unkn 1”) was identified as 7,2′-dihydroxy-4′,5′-methylenedioxyisoflav-3-ene (**7**) (Fig. 1B); it had mobility (R_f = 0.72) on TLC plates,

Table 1
Efficiency of incorporation of various isoflavonoids into (+) pisatin (**5**) in vivo[A]

% Incorporation by experiment						
[3H] Precursor[B]	(Time (h) of $CuCl_2$ added, time of substrate administration, length of administration period)					
	A(0, 0, 4)	B(0, 0, 12)	C(0, 12, 4)	D(0, 0, 12)	E(0, 1, 16)	F(0, 3.5, 26)
DMD (**1**)			1.16 ± 0.48ab			
(−) SOP (**2b**)	1.58 ± 0.44a	2.63 ± 0.25a	2.77 ± 0.67a	1.94 ± 0.26a	1.37 ± 0.18a	2.24 ± 0.51a
(+) SOP (**2a**)	0.92 ± 0.01a,b	0.65 ± 0.11b	1.23 ± 0.22b	0.85 ± 0.08b	0.45 ± 0.07c	0.81 ± 0.62b
(+) MAA (**3a**)	0.32 ± 0.01b	1.43 ± 0.19b	1.38 ± 0.25ab	1.18 ± 0.08b	0.59 ± 0.10b,c	2.89 ± 0.28a
(−) MAA (**3b**)	0.08 ± 0.01b	1.09 ± 0.43b	0.15 ± 0.00b	0.21 ± 0.05c		

Data are the means of the percent incorporation into (+) pisatin (**5**), (±) the standard error. Means with different lower case letters are significantly different at $p = <0.05$.

(A) Two cotyledon cores per sample; total, 4 per precursor; 7.5 nmol substrate administered at the time of pisatin (**5**) induction in 2% DMSO/5 mM $CuCl_2$; feeding period, 4 h. (B) Same as A except the feeding period, 12 h. (C) Same as A except the substrates were administered 12 h after induction for 4 h. (D) 1 cotyledon section per sample, total, 6 per precursor; 15 nmol substrate fed at the time of pisatin (**5**) induction in 2% DMSO/5 mM $CuCl_2$; feeding period, 12 h. (E) 1 cotyledon core per sample, total, 5 per precursor; 5 nmol substrate in 8 μL EtOH fed to tissue 1 h after pisatin (**5**) induction for 1 h; feeding period, 16 h. (F) 2 cotyledon sections per sample, total, 6 per precursor; 10 nmol substrate fed 3.5 h after pisatin (**5**) induction; feeding period, 26 h.

[A] Pisatin was identified as (+) pisatin (**5**) by HPLC on a chiral column.

[B] DMD, 7,2′-dihydroxy4′,5′-methylenedioxyisoflavone (**1**), SOP, sophorol (**2**) and MAA, maackiain (**3**).

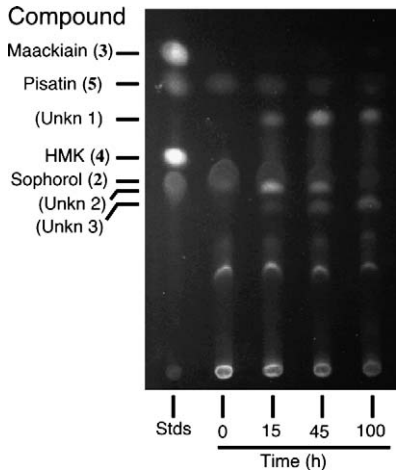

Fig. 4. Thin layer chromatogram (photographed under UV) of products produced when (−) sophorol (**2b**) is incubated with crude protein extracts from pea seedlings which had been induced to synthesize pisatin (**5**). All reactions contained 50 μM sophorol (**2**), 2 mM NADPH, pH 6.7 and were carried out at 30 °C as described in Section 4. Reactions were stopped at the indicated times. Included as standards were: maackiain (**3**), pisatin (**5**), HMK, 6a-hydroxymaakiain (**4**), and sophorol (**2**). The protein extracts from pea contained a small amount of pisatin (**5**).

HPLC retention time (R_t), UV spectrum, and mass spectrum as for the authentic standard (see Section 4). The second compound (“unkn 2”) occurred transiently and was detected by TLC only in reaction products from early time points (Fig. 4). This compound had the same fluorescence color under UV light and TLC mobility as 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol (**6**) (Fig. 1A). This is the expected product if pea has an (−) isoflavanone reductase equivalent to VR. A third compound (“unkn” 3) (Fig. 4) was not definitively identified. The production of all three compounds from (−) sophorol (**2b**) was dependent on the presence of NADPH. The compounds were not produced when (+) sophorol (**2a**) was substrate, although there was a slight reduction in the amount of (+) sophorol (**2a**) recovered from the cell free extracts after the incubation period (data not shown).

2.3. Cloning of a (−) isoflavonone reductase from pea

The radiolabeling studies and the activity of the cell free extracts of $CuCl_2$-treated pea tissue, are consistent with (−) sophorol (**2b**) as an intermediate in the biosynthesis of pisatin (**5**). Therefore an attempt was made to isolate a pea gene encoding an enzyme, which would be analogus to *Vr* and reduce (−) sophorol (**2b**). The *Vr* cDNA from alfalfa (Guo and Paiva, 1995) was used as a heterologous probe to screen a cDNA library made from pea seedlings, which had been infected with *Nectria haematococca*. Several clones hybridized strongly to the *Vr* probe and the insert of one of these (p11-1) was sequenced. The 1241-bp insert of p11-1 encodes a 978-bp open reading frame, and includes putative translation start and stop sites. The nucleotide sequence of the open reading frame is 83.5% identical to the *Vr* gene of alfalfa. The deduced amino acid sequence of the pea (−) isoflavanone reductase gene, which is called sophorol reductase (*Sor*), is 86.5% identical and 92.0% similar to *Vr* (Fig. 5).

2.4. Northern hybridization analysis

If *Sor* is involved in pisatin (**5**) biosynthesis, the induction of this gene would be expected to correlate with its production and the expression of other genes, such as *Ifr* and *Hmm*, known to be involved in pisatin (**5**) biosynthesis. Four-day old pea seedlings were treated with $CuCl_2$ to induce pisatin (**5**) biosynthesis and total RNA was isolated at various times after the treatment. The *Sor* probe hybridized with a RNA of approximately 1.35 kb (Fig. 6). A small amount of *Sor*-hybridizing RNA was present prior to $CuCl_2$ treatment (lane 1) but increased significantly at later

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alfalfa   1 MAEGKGRVCVTGGTGFLGSWIIKSLLENGYSVNTTIRADPERKRDVSFLT  50
pea       1 MAEGKGRVCVTGGTGFLGSWIIKSLLENGYSVNTTVRPDPKRKRDLSFLT  50

alfalfa  51 NLPGASEKLHFFNADLSNPDSFAAAIEGCVGIFHTASPIDFAVSEPEEIV 100
pea      51 NLPGASERLHFFNADLSDPKSFSAAVKGCVGVFHTATPVNFAVSEPEKIV 100

alfalfa 101 TKRTVDGALGILKACVNSKTVKRFIYTSSGSAVSFNGKDKDVLDESDWSD 150
pea     101 TKRTVDGALGILKACVNSKTLKRFIYTSSGSAVSFNGKNKEVLDETDWSD 150

alfalfa 151 VDLLRSVKPFGWNYAVSKTLAEKAVLEFGEQNGIDVVTLILPFIVGRFVC 200
pea     151 VGLLKSVKPFGWSYSVSKTLAEKAVLDFGQQNGIDVATLILPFIVGSFIC 200

alfalfa 201 PKLPDSIEKALVLVLGKKEQIGVTRFHMVHVDDVARAHIYLLENSVPGGR 250
pea     201 PKLPDSVDKALVLVLGKKEQIGVTRFHMVHVDDVARAHIYLLENSVPGGR 250

alfalfa 251 YNCSPFIVPIEEMSQLLSAKYPEYQILTVDELKEIKGARLPDLNTKKLVD 300
pea     251 YNCAPFIVTIEEMSQLFSAKYPEFQILSVDELKEIKGARLAELNSKKLVA 300

alfalfa 301 AGFDFKYTIEDMFDDAIQCCKEKGYL 326
pea     301 AGFEFKYSVDDMFEDAIQCCKEKGYL 326
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Fig. 5. Comparison of the deduced amino acid sequences from the sophorol reductase from pea and the vestitone reductase from alfalfa. Identical amino acids are shaded.

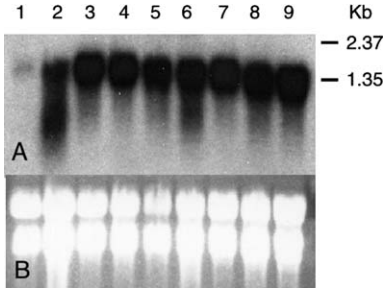

Fig. 6. Northern hybridization analysis of *Sor* expression in pea. Total RNA from pea seedlings was resolved in a formaldehyde gel, transferred to Hybond-N^+ membrane, and hybridized to ^{32}P-labeled insert from p3B1, a cDNA encoding SOR. (A) Autoradiogram, (B) the formaldehyde gel stained with ethidium bromide. Lane 1: no $CuCl_2$ treatment, lanes 2–9: 3, 6, 9, 12, 18, 21, 24, and 30 h post $CuCl_2$ treatment, respectively. Size standards (kb) are indicated on the right side in A.

time points, a pattern of accumulation which coincides with pisatin (**5**) production and the accumulation of *Ifr* and *Hmm* transcripts in $CuCl_2$ – elicited pea tissue (Paiva et al., 1994; Wu et al., 1997).

2.5. Genomic analysis

The *Sor* cDNA contains one site each for *Eco*RI and *Hin*dIII, and two *Dra*I sites. There are no *Bam*HI sites in the cDNA. In Southern hybridization analysis of genomic DNA digested by *Eco*RI, *Hin*dIII, *Eco*RI/*Hin*dIII, *Dra*I and *Bam*HI, a single DNA fragment hybridized in the lane containing the *Bam*HI digested DNA (Fig. 7), consistent with one genomic copy of *Sor*. A strongly hybridizing ~800-bp *Dra*I fragment is consistent with the predicted size of an internal *Dra*I fragment from the cDNA (819 bp) and the hybridization pattern of the *Hin*dIII and *Eco*RI/*Hin*dIII digests is consistent with the presence of *Hin*dIII and *Eco*RI sites detected in the cDNA. The reason why only

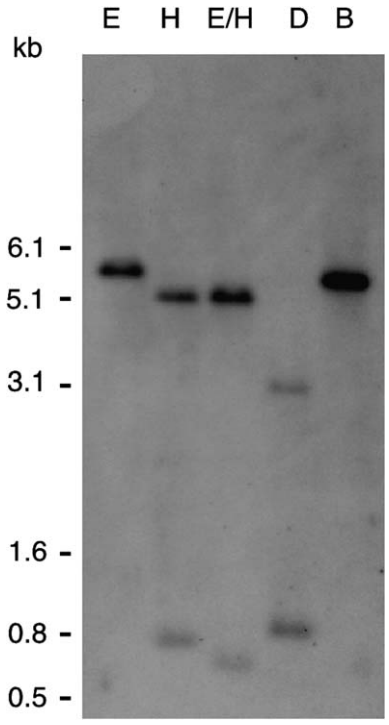

Fig. 7. Southern hybridization analysis of pea genomic DNA. Genomic DNA from pea seedlings was digested with the restriction enzymes *Eco*RI (lane 1), *Hin*dIII (lane 2), *Eco*RI and *Hin*dIII (lane 3), *Dra*I (lane 4), and *Bam*HI (lane 5), transferred to nylon membrane, and hybridized with ^{32}P-labeled p3B1 insert, a cDNA encoding SOR.

one, rather than the two fragments expected, hybridized to *Eco*RI-digested DNA is unknown, but the results are consistent with a single copy of *Sor* in pea.

Primers specific to the 5′ translational start and 3′ termination sites as well as to two internal sequences of the cDNA were used to amplify DNA specific to the *Sor* gene from genomic DNA and cloned cDNA. The PCR products

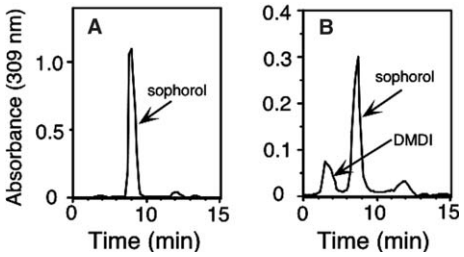

Fig. 8. Metabolism of (−) sophorol (**2b**) by SOR. The cDNA contained in p3B1, which encode sophorol reductase, was expressed in *E. coli* and used to metabolize (−) sophorol (**2b**) (50 μM) in the absence (A) or presence (B) of 1 mM NADPH for 30 min at 30 °C.

made were of the same size whether the cDNA or genomic DNA served as template. It appears, therefore, that the *Sor* structural gene is identical to the coding region of the cDNA and that no introns are present.

2.6. Analysis of recombinant SOR activity

Two cloned PCR products of *Sor* (from p2B1 and p3B1) were expressed in *E. coli* and the SOR activity in the crude enzyme preparations was compared to that of the recombinant VR from alfalfa. The protein preparations from both clones catalyzed the reduction of (−) sophorol (**2b**) to 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol (DMDI) (**6**) in a NADPH dependent manner (only data from protein of p3B1 is shown in Fig. 8), identical to the activity of recombinant VR from alfalfa (data not shown). A trace amount of DMDI (**6**) was also produced by all three proteins on (+) sophorol (**2a**), which may be due to contamination of the (+) sophorol (**2a**) stock with (−) sophorol (**2b**) caused by racemization (Guo et al., 1994a).

3. Conclusions

The better incorporation of (−) sophorol (**2b**) than (+) sophorol (**2a**) into (+) pisatin (**5**) and the identification of a (−) sophorol reductase, which is induced during the production of (+) pisatin (**5**), are consistent with the involvement of (−) sophorol (**2b**) as an intermediate in the synthesis of (+) pisatin (**5**). It would appear that the step(s) responsible for the asymmetry at carbon-6 and carbon-11 in (+) pisatin (**5**) occurs after formation of DMDI (**6**) (Fig. 1, A). The optical form of the DMDI (**6**) produced from (−) sophorol (**2b**) was not identified, but the (−) isomer **6** is more likely as it is a reductive product of (−) sophorol (**2b**). Thus, in pea the synthesis of (+) pisatin (**5**) apparently involves an unexpected stereochemical reversal in which the first stereochemically active intermediates have a stereochemistry associated with the synthesis of (−) pterocarpans and the later intermediate(s) and final product have the (+) stereochemistry.

This study did not identify the steps that follow the production of DMDI (**6**). However, preliminary experiments with crude protein extracts of pea seedlings indicate that 7,2′-dihydroxy-4′,5′-methylenedioxyisoflav-3-ene (**6**) and the unidentified compound (unkn 3, Fig. 4) produced from (−) sophorol (**2b**) also are made when DMDI (**6**) is the substrate (G. DiCenzo and H. VanEtten, unpublished results). The production of 7,2′-dihydroxy-4′,5′-methylenedioxyisoflav-3-ene (**6**) is intriguing. This isoflavene is an achiral molecule which could be converted subsequently to the (+) asymmetry by hydration across the C3–C4 double bond (Fig. 1B). However, radiolabeling studies have shown that 7,2′-dihydroxy-4′,5′-methylenedioxyisoflav-3-ene (**7**) is incorporated poorly into (+) pisatin (**5**) and unlikely to be an intermediate (Banks and Dewick, 1983). In contrast, radiolabeled isoflavene is incorporated readily into coumestans and appears to be an intermediate in the synthesis of these compounds (Martin and Dewick, 1980). While further studies are required to identify the final steps in the biosynthesis of (+) pisatin (**5**), the current results support the involvement of compounds with the (−) stereochemistry as intermediates in this pathway.

4. Experimental procedures

4.1. Plant materials

Seeds of *P. sativum* L. cultivar Alaska were obtained from Royal Seed, St. Joseph, MO. Cotyledons were prepared for use in the incorporation studies in the following way: The seeds were immersed in EtOH/H_2O/5.25% sodium hypochlorite (75:15:10) for 1 h, drained, rinsed 5× with water, and left in water to imbibe for 3–4 h. After imbibition, the seeds were spread over autoclaved moist vermiculite and germinated at room temperature for 4–6 d in the dark. Cotyledons usually were removed from the seedlings when the shoots were beginning to expand and elongate. A section of cotyledon approximately 2 mm thick was prepared for $CuCl_2$-induction and [^{3}H] substrate administration by removing and discarding an ∼2-mm thick dome-shaped section from the convex portion of the cotyledon. Experiments were performed with the ca. 2 mm thick remaining section.

To produce elicited pea tissue for enzyme assays, 4–6-day-old seedlings produced as above, were immersed in 1 mM $CuCl_2$ for 1 h and incubated in the dark for 24 h. The seedlings were rinsed well and the cotyledons removed. The seedlings were frozen in N_2 and stored at −80 °C.

4.2. Radiolabelling studies

One to two sections of cotyledon were treated with 5 mM $CuCl_2$, either prior to or at the time of [^{3}H]-isoflavonoid administration. Five to 35 μl of the candidate precursor in either 2% DMSO with 5 mM $CuCl_2$ or in EtOH–H_2O (1:1) was administered to the cotyledon sections. The time of addition of the candidate precursor varied by experiment and ranged from 0 to 31 h post

induction. The tissue sections were incubated with the substrate in several ways. In some experiments, the cotyledon slice (described above in Section 4.1) was kept on water-dampened Whatmann #4 filter paper in 35 mm polystyrene petri dishes. In others, the cotyledons were trimmed with a cork borer to ~5 mm diameter and placed in 1.5-ml microfuge tubes or in 16-mm glass test tubes. The incorporation of radiolabeled substrates was stopped by freezing the cotyledons at −20 °C. To prepare the frozen tissue for analysis, it was macerated with a glass rod in 1 ml of 1 M Tris, pH 7.5 and the slurry extracted 2× with EtOAc. The organic phases from each treatment were combined and concentrated under N_2 or in a Speed Vac Concentrator (Savant Instruments Inc, Farmingdale, NY). Pisatin (**5**) was separated from the other components in the extract by silica gel TLC and reversed phase HPLC and analyzed for incorporation of the radiolabel.

4.3. Chemicals

(+) and (−) Maackiain (**3a**) and (**3b**), (+) and (−) pisatin (**5**) and [^{14}C] pisatin were from laboratory stocks prepared as described previously (George et al., 1998). DMD (7,2′-dihydroxy-4′,5′-methylenedioxyisoflavone) (**1**) was a gift from Yuegin Sun (Eli Lilly and Company, Indianapolis, IN 46285). [^{3}H] maackiain (**3**) and DMD (**1**) were made by a method based on Banks and Dewick (1983b) as follows: 25 mg (+) or (−) maackiain (**3a**) or (**3b**) or DMD (**1**) was dissolved in 300 μl dimethylformamide to which was added 100 μl ^3H_2O (500 mCi, Amersham) and 9.8 μl triethanolamine. The solution was heated to 80 °C for ~48 h after which it was acidified with 70 μl of 1 M HCl. The solution was extracted with $CHCl_3$ (2 × 1 mL) and the combined $CHCl_3$ fractions were washed with H_2O (2 ml). The [^{3}H] labeled products were then purified by using the following solvent systems sequentially: (1) hexane–EtOAc, 3:2; (2) hexane–EtOAc–MeOH, 60:40:1; and (3) hexane–Me_2CO 2:1. While not determined in our experiments, Banks and Dewick (1983b) showed that this protocol yields maackiain (**3**) with the majority of the [^{3}H] on C4.

(+) and (−) Sophorol (**2a**) and (**2b**) were obtained by biotransformation of (+) and (−) maackiain (**3a**) and (**3b**), respectively, by *Colletotrechum trifolii* (VanEtten lab isolate T-456). Glucose-asparagine (GA) medium (100 ml) (Matthews et al., 1987) was inoculated with 0.3 g fresh weight (f.wt.) mycelia and conidia from an overnight GA culture, which had been started from cultures of *C. trifolii* grown on V-8 (Miller, 1955) agar slants. Maackiain (**3**) was added to a final concentration of 30 μM and the culture incubated at 25 °C on a shaker (175 rpm). After 5–6 h, the culture was extracted 2× with equal volumes of EtOAc. The organic layers were combined and concentrated in a Büchi RE121 rotary evaporator (Büchi, Flawil Switzerland). Sophorol (**2**) was separated from maackiain (**3**) and 1a-hydroxymaackiain (another product of maackiain metabolism) by silica gel TLC in toluene:EtOAc (60:40).

7,2′-Dihydroxy-4′,5′-methylenedioxyisoflavonol (DMDI) (**6**) was made from (−) sophorol (**2b**) by the method of Guo and Paiva (1995). Briefly, a culture of *E. coli* DH5α harboring the pVR1 cDNA in the expression vector pSE380 was grown at 37 °C to an OD of 0.6 (A_{600}). The expression of the recombinant *VR* was induced with 5 mM isopropyl-β-D-thiogalactopyranoside and the culture grown for an additional 2 h at 37°C. A reaction mixture (500 μl total volume) containing 50 μl cell lysate with 50 μM (−) sophorol (**2b**), 1 mM NADPH in 0.2 mM phosphate pH 6.0 was incubated at 30 °C for 15 min; a longer period of incubation did not increase the yield of DMDI (**6**). DMDI (**6**) was separated from the substrate by reversed phase HPLC (see below). 7,2′-Dihydroxy-4′,5′-methylenedioxyisoflav-3-ene (**7**) was made following the method of Banks and Dewick (1983b). β-NADPH was from Sigma Chemical Company, St. Louis, MO.

4.4. Analysis of isoflavanone metabolites by TLC and HPLC

TLC and autoradiography were carried out essentially as described by Preisig et al. (1990). The extracts were applied to silica gel TLC plates (EM Sciences Silica gGel 60 F_{254}, aluminum backed) and developed in toluene:EtOAc, 60:40. The developed plates were photographed under both long and short wavelength UV light to record the locations of the compounds. Autoradiography was carried out by coating the developed plate with a 10% solution of 2,5-diphenyloxazole (PPO) in Et_2O. The solution was applied by painting the plate with a wad of silanized glass wool dipped in the liquid. After drying, the plate was wrapped in polyethylene film and enclosed in an X-ray cassette with Kodak X-Omat film (Kodak, Rochester, NY). The X-ray film was exposed at −80 °C for 2–3 weeks before processing. In some experiments, the compounds detected under both long and short wavelength UV light, were extracted from the silica gel with EtOH. Their UV absorbance spectra was measured with a Beckman (Fullerton, CA) DU-64 scanning spectrophotometer.

Reversed phase HPLC (RP-HPLC) used a gradient system (24–74% CH_3CN in H_2O). Normal phase HPLC was performed isocratically using $MeCl_2$–EtOAc–EtOH–AcOH (100:4.5:0.5:0.5). Both RP-HPLC and normal phase HPLC runs were monitored at 309 and 278 nm. The UV absorbance of selected compounds was measured as above.

Chiral chromatography was performed isocratically on a ChiralPak OT+ column (J.T. Baker, Phillipsburg, PA) (2.5 × 250 mm) in MeOH at 0.5 ml/min at 5 °C. Absorbance was monitored at 309 and 278 nm. In this system, the R_t for (−) pisatin was ~9.3 and 10.2 min for (+) pisatin (**5**).

4.5. Analysis of isoflavanone metabolites by MS

The mass spectra of HPLC-purified samples were determined on an HP 5988A using a direct insertion probe, a

temperature range of 30–350 °C (ramped 30°/min) and an EI of 70 eV. Alternatively, direct inlet was used on a Jeol HX-110A Sector mass spectrometer in either CI+ mode with iso-butane, or in EI+ mode. Derivatization was performed using bis (Trimethyl-Sylil) Triflouroacetamide (BSTFA) (Sigma) essentially according to the manufacturer's instructions: the sample was dissolved in 14 μl pyridine, 7 μl BSTFA were added and the mixture heated to 70 °C for 15 min.

4.6. Chemical properties of enzymatic products

7,2′-Dihydroxy-4′,5′-methylenedioxyisoflav-3-ene (**7**) had the broad UV absorbance maximum centered at ~338 nm, and a GC–MS spectrum with major ions at $m/z = 283$ (22%) and $m/z = 284$ (100%). The trimethylsylil (TMS)-derivative yielded the expected molecular ion at $m/z = 428$ (284 + 2 TMS). All of these values also were obtained with the authentic standard.

4.7. Isolation of the cDNA of Sor

A cDNA library (7.5×10^4 recombinants) of poly (A^+) RNA from pea tissue infected with *N. haematococca* in the vector Uni-ZAP XR (Stratagene) (Han et al., 2001) was screened by in situ plaque-hybridization with the insert of pVR1 (gift of Nancy Paiva, Nobel Foundation, Ardmore OK). Hybridization was performed at 65 °C. The blots were washed at 65 °C with 1× SSC, 0.1% SDS for 45 min, and with 0.1× SSC, 0.1% SDS for 30 min. Fourteen strongly-hybridizing plaques were subjected to a second round of screening. The cDNA inserts were rescued in pBluescript SK(−) following the in vivo excision protocol for λZAP (Stratagene). DNA from each clone was digested with *Eco*RI and *XhoI* and subjected to Southern analysis using the insert of pVR1 as a probe. Several clones had a strongly hybridizing 0.75-kb *Eco*RI fragment and a 0.5-kb *Eco*RI/*Xho*I fragment, but also contained additional insert DNA, suggestive of concatenated cDNAs. The 0.75- and 0.50-kb fragments from p11-1 were gel-purified using the Qiaex II system (Qiagen, Santa Clarita, CA) and ligated into pBS digested with *Eco*RI and *Xho*I. The 0.75-kb and the 0.50-kb inserts in the resulting plasmids and the 5′ and 3′ ends of the insert in p11-1were sequenced by DNA Sequencing Service, Arizona Research Laboratories, University of Arizona. The sequence of the region spanning the junction between the 0.75- and 0.05-kb fragments was obtained from p11-1. The sequence of the full-length cDNA was assembled from the partial sequences (Genbank Accession Number AF107404).

In order to clone a full-length homologue of the pVR1 cDNA from p11-1, synthetic primers (GibcoBRL) that were specific to the predicted 5′ and 3′ ends of the cDNA and that introduced unique restriction sites were utilized. A *Nco*I site was introduced at the 5′ end of the cDNA using (5′-ACG CCA TGG CAG AGG GGA AAG GAA GGG T-3′). Similarly, a *Bam*HI site was introduced at the 3′ end using (5′-ACG CGG ATC CTT AGA GAT ATC CTT TTT CCT-3′). Each PCR reaction contained PCR reaction buffer, 40 μl H_2O, each dNTP [200 μM], $MgCl_2$ [1.5 mM], primers [1.0 μM], 20 ng p11-1 (template), and 1.25 U Taq polymerase (GibcoBRL) in a total volume of 50 μl. The program was as follows: 2 min denaturation at 95 °C, followed by 35 cycles of: denaturation (95 °C, 1 min), annealing (60 °C, 1 min), and extension (72 °C, 2 min). The combination of primers produced an ~1-kb DNA fragment, which was gel-purified, extracted with phenol–$CHCl_3$, and precipitated with 1/10 vol. of 3 M NaOAc + 2 vol. of 100% EtOH. Two such PCR products, identified as pcr 2B and pcr 3B, were purified and each was double-digested with *Nco*I and *Bam*HI. The expression vector pSE380 was similarly digested and, after gel-purification of the PCR products and the vector using the Qiaex II system, the PCR fragments were ligated into pSE380. Two clones, p2B1 and p3B1, containing PCR products 2B and 3B, respectively, were selected for further analysis.

4.8. Northern analysis

Pea seedlings were treated with $CuCl_2$ for 1 h and subsequently incubated for various lengths of time. RNA was isolated from $CuCl_2$-induced seeding using TRIZOL Reagent (GibcoBRL Life Technologies, Gaithersburg, MD) following the manufacturer's instructions. Electrophoresis of isolated RNA in 1% agarose/formaldehyde gels was performed as described by Ausubel et al. (1993). The RNA was transferred onto nylon membranes (Hybond N^+ Amersham Biosciences Co., Piscataway, NJ), and hybridized with ^{3}P-labeled *Sor* obtained from p3B1. The hybridization and the washing of the membrane were as described for the isolation of the *Sor* cDNA.

4.9. Southern hybridization

Total genomic DNA was purified from 30 g of pea seedlings as described Saghai-Maroof et al. (1984). Genomic DNA (100 μg) was digested with restriction enzymes, subjected to electrophoresis in an 0.8% agarose gel and transferred onto nylon membrane (Hybond-N^+). The blotted DNA was prehybridized for 1 h at 65 °C and subsequently hybridized with the ^{32}P-labeled probe for an additional 12–18 h at 65 °C. The membranes were washed as described for the isolation of the *Sor* cDNA.

4.10. PCR analysis

PCR amplification of genomic DNA was compared to that of the cDNA by using several combinations of primers: the primers specific to the 5′ and 3′ ends of the deduced coding region (described above) were used to amplify a 992-bp fragment of the cDNA and the corresponding region from genomic DNA. The 5′ primer also was combined with an internal downstream primer 852 bp after the start of the cDNA coding region. Likewise, the primer

specific to the 3′ end of the gene was combined with an upstream primer starting 688 bp from the ATG at the start of the cDNA coding region. Both the p11-1 cDNA and genomic DNA were used as templates in order to detect sequence differences such as might arise from an intron.

4.11. Preparation of ^{32}P-labeled probes

^{32}P-labeled inserts of pVR1 cDNA were made using the Prime-It II Random Labeling Kit (GibcoBRL). The ^{32}P-labeled probes were column-purified by elution through Sephadex G 50.

4.12. Expression of isoflavanone reductase (Sor) activity in E. coli

Recombinant SOR was produced and its activity measured as described above for the production of DMDI by the *Vr* cDNA clone from alfalfa.

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