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Inactivation of pea genes by RNAi supports the involvement of two similar *O*-methyltransferases in the biosynthesis of (+)-pisatin and of chiral intermediates with a configuration opposite that found in (+)-pisatin

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Abstract

(+)-Pisatin, the major phytoalexin of pea (*Pisum sativum* L.), is believed to be synthesized via two chiral intermediates, (−)-7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanone [(−)-sophorol] and (−)-7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol [(−)-DMDI]; both have an opposite C-3 absolute configuration to that found at C-6a in (+)-pisatin. The expression of isoflavone reductase (IFR), which converts 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavone (DMD) to (−)-sophorol, sophorol reductase (SOR), which converts (−)-sophorol to (−)-DMDI, and hydroxymaackiain-3-*O*-methyltransferase (HMM), believed to be the last step of (+)-pisatin biosynthesis, were inactivated by RNA-mediated genetic interference (RNAi) in pea hairy roots. Some hairy root lines containing RNAi constructs of IFR and SOR accumulated DMD or (−)-sophorol, respectively, and were deficient in (+)-pisatin biosynthesis supporting the involvement of chiral intermediates with a configuration opposite to that found in (+)-pisatin in the biosynthesis of (+)-pisatin. Pea proteins also converted (−)-DMDI to an achiral isoflavene suggesting that an isoflavene might be the intermediate through which the configuration is changed to that found in (+)-pisatin. Hairy roots containing RNAi constructs of HMM also were deficient in (+)-pisatin biosynthesis, but did not accumulate (+)-6a-hydroxymaackiain, the proposed precursor to (+)-pisatin. Instead, 2,7,4′-trihydroxyisoflavanone (TIF), daidzein, isoformononetin, and liquiritigenin accumulated. HMM has a high amino acid similarity to hydroxyisoflavanone-4′-*O*-methyltransferase (HI4′*O*MT), an enzyme that methylates TIF, an early intermediate in the isoflavonoid pathway. The accumulation of these four compounds is consistent with the blockage of the synthesis of (+)-pisatin at the HI4′*O*MT catalyzed step resulting in the accumulation of liquiritigenin and TIF and the diversion of the pathway to produce daidzein and isoformononetin, compounds not normally made by pea. Previous results have identified two highly similar "HMMs" in pea. The current results suggest that both of these *O*-methyltransferases are involved in (+)-pisatin biosynthesis and that one functions early in the pathway as HI4′*O*MT and the second acts at the terminal step of the pathway.

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

1. Introduction

(+)-Pisatin **8** (see Fig. 1), a pterocarpanoid phytoalexin synthesized by pea (*Pisum sativum* L), was the first phytoalexin whose chemical structure was identified (Cruickshank and Perrin, 1960; Perrin and Bottomley, 1962). Significant progress towards elucidating the pathway for its biosynthesis has been made through biochemical studies in pea and related legumes (Dewick, 1988,1994; Dixon, 1999). The basic pterocarpan carbon skeleton contains four rings ABCD as shown for (+)-pisatin **8** in Fig. 1. Because of the presence

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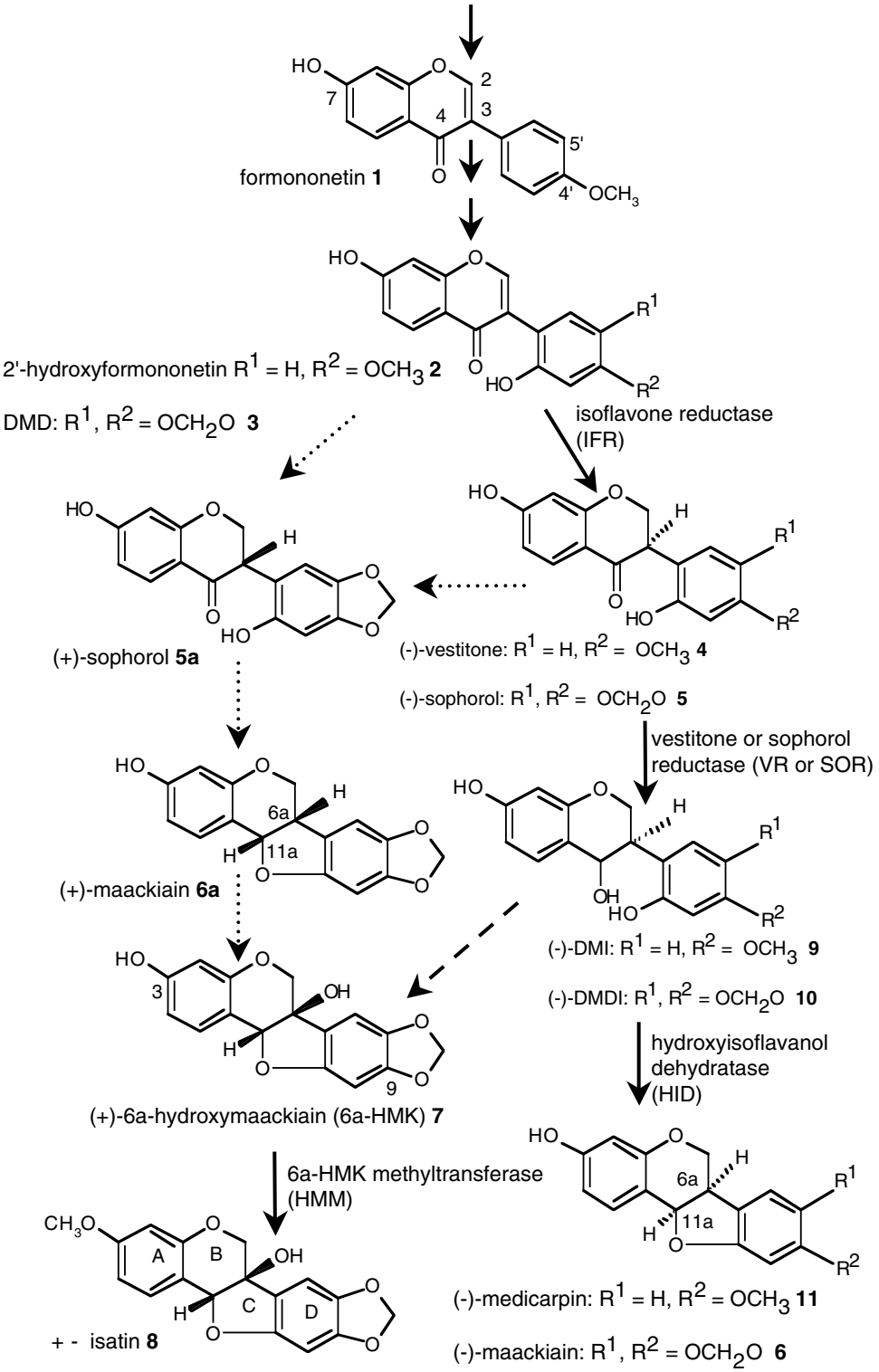

Fig. 1. Scheme for biosynthesis of the major pterocarpans of chick pea, alfalfa and pea. Solid arrows represent the steps in which enzymes have been identified for the biosynthesis of (+)-pisatin **8** in pea, (−)-medicarpin **11** in alfalfa and (−)-maackiain **6** in chickpea, while dashed arrows represent a proposal (Banks and Dewick, 1982a,b; Banks et al., 1983) for the biosynthesis of (+)-pisatin **8** through (+)-sophorol **5a**, and (+)-6a-hydroxymaackiain **7**. The dotted line is the step(s) at which it is proposed intermediate(s) are formed so that the oxygen in the 6a hydroxy of (+)-pisatin **8** is derived from water. DMD = 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavone **3**, DMI = 7,2′-dihydroxy-4′-methoxy-isoflavanol **9**, DMDI = 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol **10**.

of sterogenic centers at the adjacent 6a and 11a positions in pterocarpans, four stereoisomers are possible, but only two forms, exemplified by (+)-6a and (−)-maackiain **6** (Fig. 1), are known to occur in nature.[1] While most legumes produce pterocarpan phytoalexins with an absolute configuration as shown for (−)-medicarpin **11** and (−)-maackiain **6** (Fig. 1), the garden pea is somewhat unusual in that it synthesizes a (+)-pterocarpan, (+)-pisatin **8** (Fig. 1) (Banks and Dewick, 1982b; Dewick, 1988).

In 1982, Banks and Dewick (1982a,b) suggested that the biosynthesis of (+)-pisatin **8** proceeded via intermediates such as (+)-sophorol **5a** and (+)-maackiain **6a**, which have stereogenic centers with the same configuration as found in (+)-pisatin **8** (Fig. 1). However, recent studies have suggested that the pathway, up to the (−)-7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol [(−)-DMDI **10**] intermediate, involve chiral intermediates with the opposite absolute configuration at what will become carbon 6a in (+)-pisatin **8** (DiCenzo and VanEtten, 2006). Specifically, the isoflavone reductase (IFR) catalyzing the conversion of 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavone (DMD **3**) to (−)-sophorol **5** in pea (Fig. 1) has been characterized and a cDNA for it isolated (Paiva et al., 1994). This IFR, like those from alfalfa (Paiva et al., 1991), chickpea (Schlieper et al., 1990), and soybean (Fischer et al., 1990), which make (−)-pterocarpans, converted the achiral isoflavone to a chiral (−)-isoflavanone with the configuration at C-3 as shown for (−)-sophorol **5** (Fig. 1). There was no evidence for the existence of an IFR that produces (+)-sophorol **5a** from the achiral intermediate, DMD **3**, or of an epimerase that might produce (+)-sophorol **5a** from (−)-sophorol **5** in the protein extracts of (+)-pisatin-synthesizing pea tissue (Paiva et al., 1991; DiCenzo and VanEtten, 2006). In addition, precursor administration studies using radiolabelled intermediates have demonstrated that (−)-sophorol **5** is incorporated into (+)-pisatin **8** better than either (+)-sophorol **5a** or (+)-maackiain **6a** (Dicenzo and VanEtten, 2006). Furthermore, a sophorol reductase, (SOR) that prefers (−)-sophorol **5** over (+)-sophorol **5a** as a substrate, was identified in pea tissue synthesizing (+)-pisatin **8** (DiCenzo and VanEtten, 2006). These results support a (+)-pisatin **8** biosynthetic pathway that proceeds through chiral intermediates with a configuration opposite to that found in (+)-pisatin **8** (Fig. 1).

The current model for the late steps for (+)-pisatin **8** biosynthesis includes (−)-sophorol **5** and (−)-DMDI **10** as intermediates and has (+)-6a-hydroxymaackiain [(+)-6a-HMK **7**] as the immediate precursor of (+)-pisatin **8**. The biochemical basis for the conversion of (−)-DMDI **10** to (+)-6a-HMK **7**, an intermediate with the opposite configuration at C-6a, remains to be elucidated, but the oxygen of the C-6a hydroxy group of (+)-pisatin **8** is known to come from water (Matthews et al., 1987,1989).

Although data from early research supports the involvement of the identified IFR, SOR and HMM pea genes in (+)-pisatin **8** biosynthesis, pea also has the capacity to synthesize (−)-maackiain **6** and (−)-medicarpin **11** derivatives (Stoessl, 1972; Pueppke and VanEtten, 1976) and the identified IFR and SOR enzymes might be involved only in the biosynthesis of these pterocarpans. In the current study, RNA-mediated genetic interference (RNAi) (Waterhouse et al., 1998,2001; Watson et al., 2005) in transgenic pea hairy roots was used to directly test the involvement of IFR, SOR and HMM in (+)-pisatin **8** biosynthesis. In addition, pea tissue was examined for the presence of an enzymatic activity capable of converting (−)-DMDI **10** to precursors of (+)-6a-HMK **7**. These experiments were done with transgenic pea hairy roots because of the rapidity and ease of producing transgenic tissue compared to the time and effort required to produce transgenic pea plants (Hamill and Lidgett, 1997; Wu and VanEtten, 2004).

2. Results

2.1. Production of pea hairy root cultures

Wild type pea hairy roots (designated R1000ni) were produced by infecting pea stem tissue with *Agrobacterium rhizogenes* strain R1000 as described previously (Wu and VanEtten, 2004). RNAi vectors consisting of two copies of cDNA of IFR, SOR or HMM arranged as an inverted repeat in the binary vector pFGC5941 were constructed. The resulting vectors were used to produce hairy roots containing the RNAi constructs. One hundred eighteen, twenty-eight, and eight independent hairy root lines transformed with the RNAi constructs with inverted cDNA copies of HMM, SOR, and IFR, respectively, were obtained. The yield of hairy root lines with the RNAi constructs of IFR, as measured by the number of hairy roots obtained per total number of pea stems inoculated with *A. rhizogenes* was low (4%) compared to that obtained with the HMM RNAi constructs (65%) or the SOR RNAi constructs (18%).

2.2. Time course analysis of (+)-pisatin biosynthesis in wild type and RNAi pea hairy root lines

Pea hairy roots without an RNAi construct (R1000ni) were treated with 0.08 mM $CuCl_2$ to elicit (+)-pisatin **8** production and the quantities produced were monitored at 24 h intervals up to 72 h after elicitation. Non-elicited wild type hairy root tissue, R1000ni, produced a mean of 5.6 μg/gFW of (+)-pisatin **8** ($T = 0$), and there was a

[1] For simplicity, the configurations of pterocarpans are commonly specified by their sign of rotation since the *R*/*S* designation is affected by the influence of the substituents on the CIP-sequence rules. For example, (+)-pisatin **8** is the (6a*R*, 11a*R*) isomer and (+)-maackiain **6a**, which has the same absolute configuration as (+)-pisatin **8,** is (6a*S*, 11a*S*) isomer because the presence of a H instead of a 6a-hydroxy changes the *R*/*S* designation. Thus, in this paper, all isoflavonoids with the absolute configurations shown for (+)-pisatin **8** will be labeled '+' and those that have the opposite will be labeled '−'.

progressive increase to 77 μg/g FW by 72 h after elicitation (Fig. 2). The same response was obtained with control hairy roots (R1000e) that contained the binary vector pFGC5941 without inserts (data not shown). Transgenic hairy roots silenced at each of the three enzymatic steps contained little or no (+)-pisatin **8** at $T = 0$. When assayed 24 h after elicitation, 23 of the 108 lines HMM RNAi constructs (HMMi lines) produced very little or no (+)-pisatin **8** (0–0.53 μg/g FW), 32 showed 50–80% reduction, and the rest showed little (10–15%) or no reduction in (+)-pisatin **8** content when compared to non-transgenic hairy root line R1000ni. Five of the transgenic lines, (HMMi #1, #3, #4, #6, and #10) from the group that produced little or no (+)-pisatin **8** were selected for further analysis. The pisatin-deficient lines and transformant HMMi #55, representing the group of transgenic hairy roots that produced reduced amounts of (+)-pisatin **8**, were compared to wild type lines for (+)-pisatin **8** production over a 72-h period (Fig. 2a). Transgenic hairy root lines HMMi #1, #3, #4, #6 and #10 consistently showed no increase in (+)-pisatin **8** content up to 72 h after elicitation with $CuCl_2$ (only data for HMMi #10 are shown), while HMMi #55 showed a slight increase in (+)-pisatin **8** (Fig. 2a) over the same period.

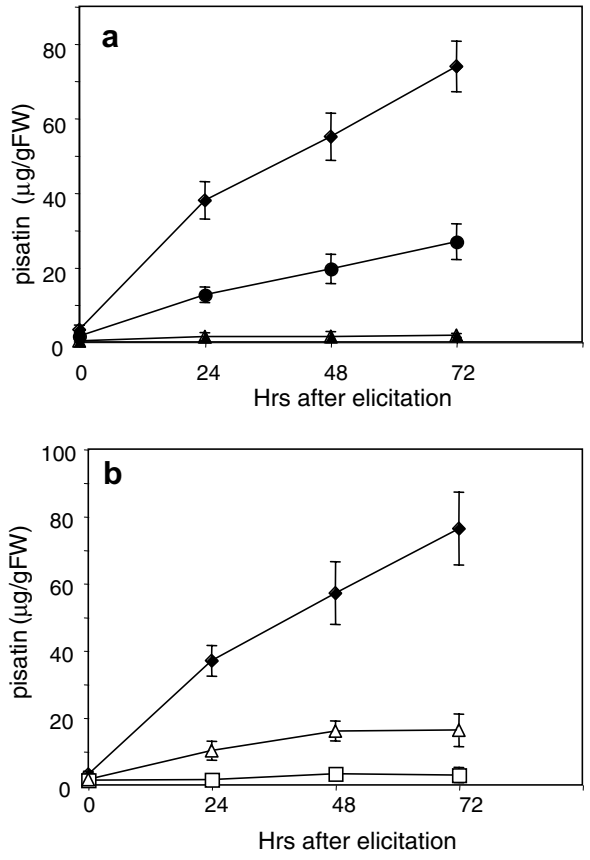

Fig. 2. Time-course analysis of the production of (+)-pisatin **8** in transgenic vs. non-transgenic pea hairy root lines after elicitation with 0.08 mM $CuCl_2$. (+)-Pisatin **8** was extracted by partitioning into hexanes and the quantities of the phytoalexin were calculated from its absorbance at 309 nm over a 72 h period. (a) (+)-Pisatin **8** produced by the control hairy root line, R1000ni produced by infecting pea stem tissue with *A. rhizogenes* (filled diamonds), transgenic hairy root lines containing RNAi constructs of HMM, HMMi #10 (filled triangles) and HMMi #55 (filled circles). (b) (+)-Pisatin **8** produced by control hairy root culture R1000ni produced by infecting pea stem tissue with *A. rhizogenes* (filled diamonds), transgenic hairy root cultures containing RNAi constructs of SOR, SORi #15 (open squares) and SORi #21 (open triangles). Error bars represent means and standard deviations of three replicate samples. All experiments were repeated 3 times with similar results.

All of the eight transgenic hairy root lines carrying the IFR RNAi construct to inactivate IFR expression (IFRi lines) produced reduced amounts of (+)-pisatin **8**, but none was completely deficient in (+)-pisatin **8** biosynthesis, when assayed 24 h after elicitation. Preliminary analysis of isoflavonoid production was completed but additional studies were not done because the cultures did not survive long enough (see below).

Of the 28 independent lines containing the SOR RNAi construct (SORi lines), five (SOR #2, #3, #6, #12, and #15) produced very little (<1 μg/g FW) or no (+)-pisatin **8** and four of them showed marked reduction in (+)-pisatin **8** production (4–15 μg/g FW) compared to controls at $T = 0$ or at 24 h after elicitation. SORi #2 and SORi #15 were selected for further analysis and in the time-course experiments, they showed very low amounts of (+)-pisatin **8** production 72 h after elicitation (Fig. 2b). SOR #21 is representative of the group that produced a reduced amount of (+)-pisatin **8**. It demonstrated an intermediate increase in the amounts of (+)-pisatin **8** over the 72 h period after elicitation.

2.3. HPLC profiles of secondary metabolites in wild type vs. RNAi pea hairy roots

To determine whether the (+)-pisatin-deficient phenotype produced changes in the metabolic profiles of pea tissue, chloroform-soluble metabolites extracted from both wild type and RNAi hairy roots were analyzed by reversed phase high performance liquid chromatography (HPLC). When monitored at 309 nm, the HPLC profiles of secondary metabolites from wild type hairy roots (R1000ni) indicated the presence of two major peaks. The main peak corresponded to a compound that had a retention time (R_t) of 15.9 min and UV absorption spectrum of (+)-pisatin **8** while the minor compound ($R_t = 16$ min) had the properties of maackiain **6** (Fig. 3a). A subsequent analysis for enantiomers demonstrated that these two compounds were (+)-pisatin **8** and (−)-maackiain **6**.

In contrast to the HPLC profile of isoflavonoid extracts from R1000ni hairy roots (Fig. 3a), chloroform extracts from HMMi #10 made 24 h after elicitation contained neither (+)-pisatin **8** nor (−)-maackiain **6** (Fig. 3b). The inactivation of HMM expression by RNAi was expected to lead to the accumulation of (+)-6a-HMK **7** (as predicted in Fig. 1). However, this was not observed in transgenic HMMi #10 or in any of the other independent lines (e.g., lines #1, #3, #4 and #6) that were silenced for HMM expression. Instead, several compounds e.g., isoformo-

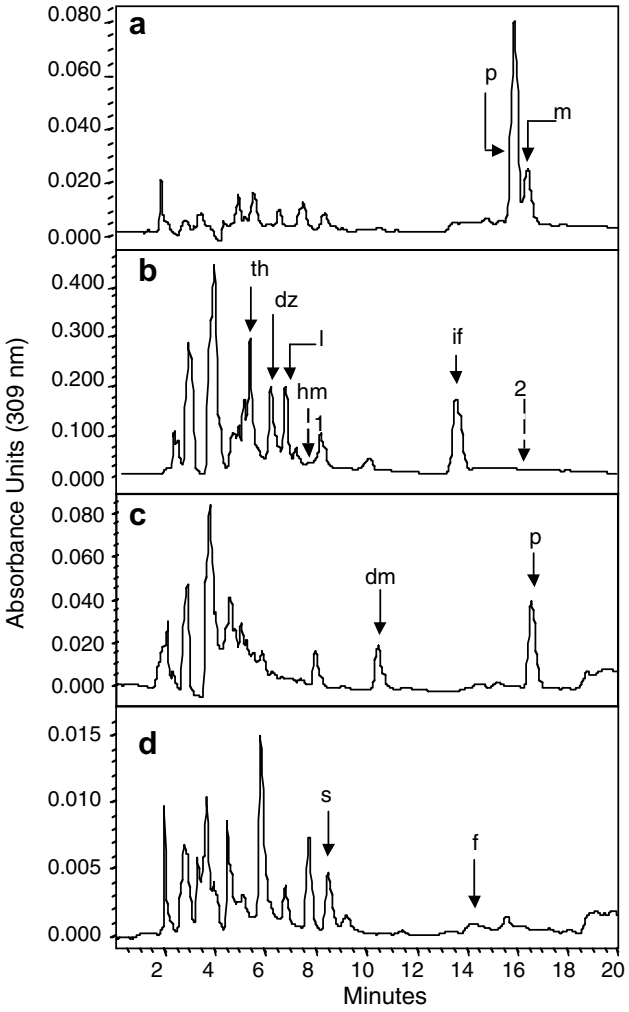

Fig. 3. HPLC profiles of secondary metabolites extracted from non-transgenic hairy roots vs. those from hairy roots containing RNAi contructs of HMM, IFR or SOR 24 h after elicitation with 0.08 mM $CuCl_2$. (a) Non-transgenic control R1000ni, (b) transgenic HMMi #10, (c) transgenic IFRi #7 and (d), transgenic SORi #2. p = (+)-pisatin **8**, m = (−)-maackiain **6**, th = 2,7,4′-trihydroxyisoflavanone 15, dz = daidzein 17, l = liquiritigenin 14, if = isoformononetin 18, s = (−)-sophorol **5**, f = formononetin **1**, hm = (+)-6a-hydroxymaackiain **7**. Dashed arrow 1 in (b) represents the retention time for (+)-6a-HMK **7**, while dashed arrow 2 indicates the retention time of (+)-pisatin **8**. Isoliquiritigenin and isoformononetin **18** co-eluted at $R_t = 13.9$ min.

nonetin **18** ($R_t = 13.9$ min, 26 μg/gFW), liquiritigenin **14** ($R_t = 6.6$ min, 17.3 μg/gFW), daidzein **17** ($R_t = 6.2$ min, 12 μg/gFW), isoliquiritigenin ($R_t = 13.92$ min, 14 μg/gFW) and 2,7,4′-trihydroxyisoflavanone **15** ($R_t = 5.5$ min) accumulated in the HMMi #10 (Fig. 3b). The other hairy roots (HMMi #1, #3, #4 and #6) that also produced very little or no (+)-pisatin **8** did not produce (+)-6a-hydroxymaackiain **7** either but did produce significant amounts of the same compounds identified in the extracts from HMMi #10.

As indicated above, it was difficult to obtain a large number of hairy root lines carrying the IFR RNAi construct suggesting that silencing of this gene had pleiotropic effects on the growth of hairy roots in addition to blocking a specific step in (+)-pisatin **8** biosynthesis. One of the IFRi hairy root lines (IFRi #7) accumulated DMD **3** (Fig. 3c), $R_t = 10.5$ min. DMD is the expected intermediate in the conversion of DMD **3** to (−)-sophorol **5** by IFR (Fig. 1) and its accumulation is consistent with the involvement of the compound and IFR in (+)-pisatin **8** biosynthesis. No further analyses of IFRi lines were carried out because of the difficulty of obtaining and maintaining them.

HPLC profiles of secondary metabolites extracted from SORi #2 (Fig. 3d) established the accumulation of (−)-sophorol **5** ($R_t = 8.45$ min, 13 μg/gFW), as expected, and formononetin **1** ($R_t = 14.4$ min, 1.1 μg/gFW), but little or no (+)-pisatin **8** or (−)-maackiain **6**. HPLC analysis of the extracts of SORi transgenic hairy root lines #2 and #15, as well as several others deficient in (+)-pisatin **8** biosynthesis, also showed several major HPLC peaks including one at $R_t = 8$ min with an UV absorption maximum at 348 nm. This compound also was observed in the extracts of HMMi hairy root lines.

2.4. Metabolism of (−)-DMDI produced from (−)-sophorol by protein extracts of pea

It was anticipated that RNAi silencing of the HMM-catalyzed step would provide insight into the nature of the intermediates that precede (+)-6a-HMK **7**. However, the lack of accumulation of the predicted intermediates raised the possibility that the pathway was not silenced at the final step (see below). Therefore, an alternative approach was taken to identify the intermediate after (−)-DMDI **10**, an intermediate believed to precede (+)-6a-HMK **7** in the (+)-pisatin **8** biosynthetic pathway. This alternative approach sought to identify an enzymatic activity capable of converting (−)-DMDI **10** to other possible intermediates in the late steps of (+)-pisatin **8** biosynthesis.

To test whether (−)-DMDI **10** could be an intermediate involved in (+)-pisatin **8** biosynthesis, (−)-DMDI **10** was first synthesized from (−)-sophorol **5**. Previous studies (Wähälä et al., 1997) have shown that the chemical reduction of isoflavones produce *cis*-**10** and *trans*-isoflavan-4-ols **10a** (Fig. 4). The reduction of (−)-sophorol **5** by $NaBH_4$ produced two major compounds ($R_t = 6.34$ and 7.0 min, respectively (Fig. 5a), both of which have the molecular mass of DMDI **10** (FW = 301) as empirically determined by Atmospheric Pressure Chemical Ionization Mass Spectrometry (APCI-MS) (data not shown). When (−)-sophorol **5** was incubated with SOR expressed in *Escherichia coli*, the two DMDI **10** isomers also were produced with the major product being the isomer at $R_t = 7$ min. The compound with $R_t = 6.34$ min has a maximum UV absorption at 300.5 nm with a shoulder at 285 nm (Fig. 5b) while the one with $R_t = 7$ min has a maximum UV absorption at 299.4 nm with a shoulder at 285 nm (Fig. 5a and b). The UV absorption spectra of both compounds were identical to that previously reported by DiCenzo (1998) for the (−)-DMDI **10** produced from (−)-sophorol **5** by the recombinant pea SOR expressed in *E. coli*.

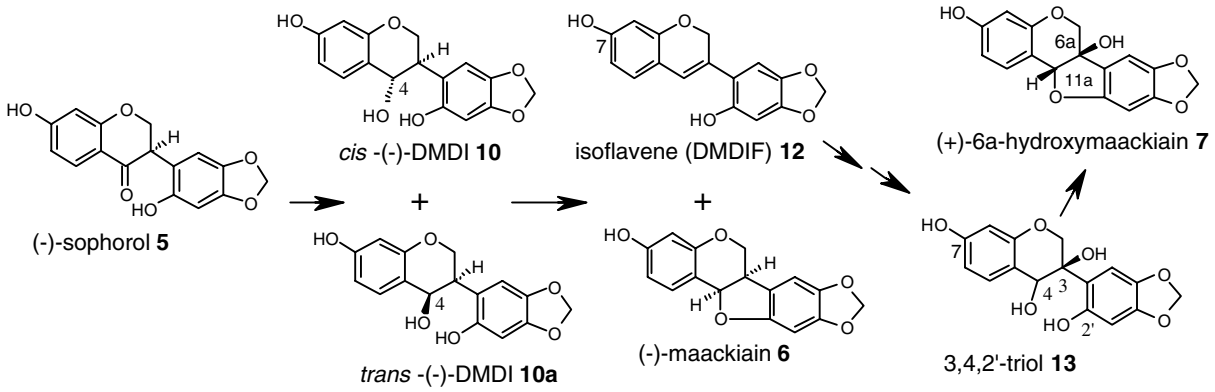

Fig. 4. Proposed mechanism by which (+)-pisatin **8** is biosynthesized from intermediates with (−)-stereochemistry via an isoflavene intermediate. The conversion of DMDIF **12** to 3,7,2′-trihydroxy-4′,5′-methylenedioxyisoflavanol (3,4,2′ triol **13**) and then (+)-6a-hydroxypterocarpan, [(+) 6a-HMK **7**] is based on the intermediates made in the chemical synthesis of 6a-hydroxypterocarpans described by van Aardt et al. (2001).

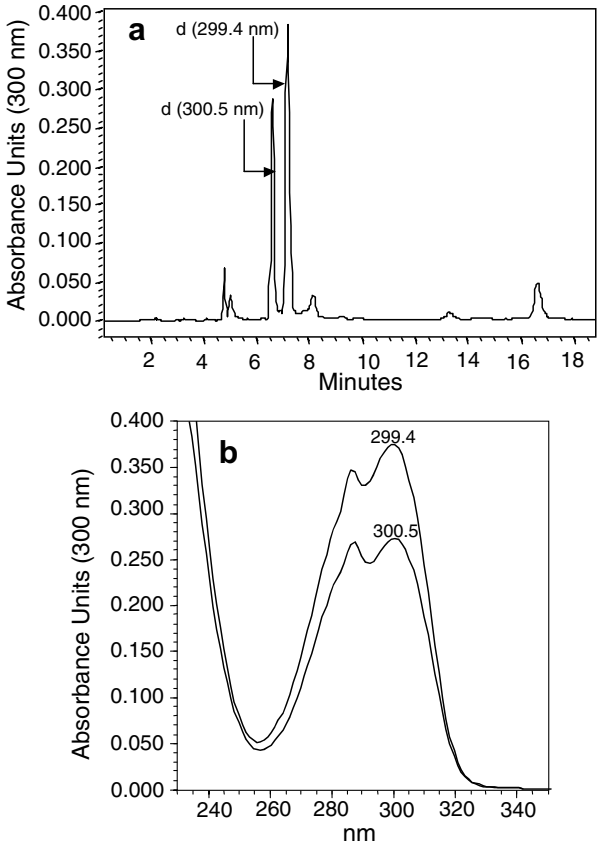

Fig. 5. Production of (−)-DMDI **10** substrate. (−)-Sophorol **5** was converted to (−)-DMDI **10** by $NaBH_4$ and products were analyzed by HPLC. A two major peaks corresponding to the two isomers (*cis* and *trans*) of (−)-DMDI **10**, **10a**. (b) UV absorption spectra corresponding to the two major peaks in (a).

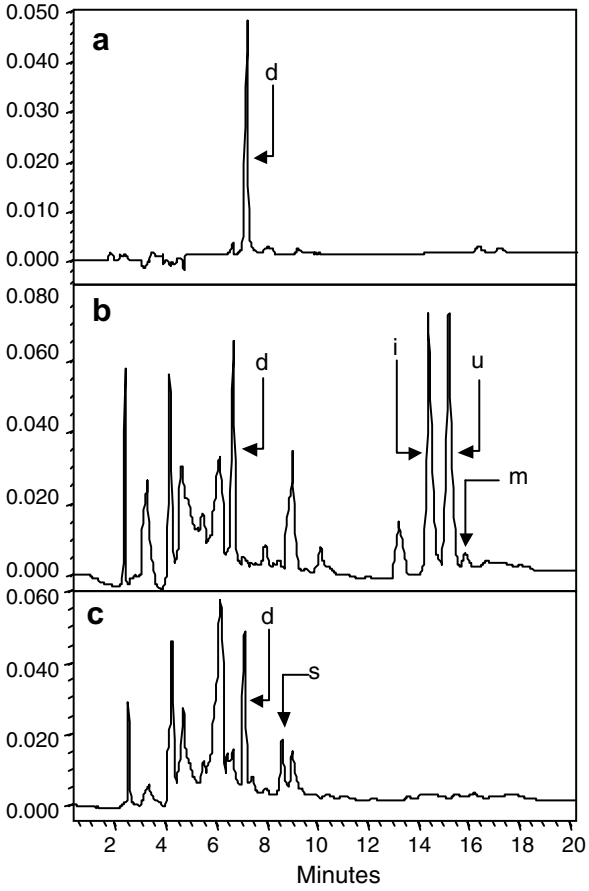

Fig. 6. Enzymatic conversion of (−)-DMDI **10** to an isoflavene by pea crude protein extracts. (−)-DMDI **10** was purified by TLC and HPLC, (a), and incubated with pea crude protein extracts for 2.5 h and the resulting products were analyzed by HPLC. (b) HPLC trace for the analysis of products obtained after the incubation of (−)-DMDI **10** with protein extracts. (c) HPLC trace for the analysis of products obtained after the incubation of (−)-DMDI **10** with boiled pea protein extracts. d = 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol 10, s = (−)-sophorol **5**, m = (−)-maackiain **6**, i = 7,2′-dihydroxy-4′,5′-methylenedioxyisoflav-3-ene **12**, u = unknown.

Although, which of the two compounds was the *cis*-**10** isomer and which was the *trans*-**10a** isomer of DMDI, was not determined in this study, the isomer produced in larger quantities (Fig. 5a, $R_t = 7$ min) by both the recombinant SOR expressed in *E. coli* and by $NaBH_4$-reduction of (−)-sophorol **5** was purified by thin layer chromatography and HPLC (Fig. 6a). This was used as a substrate for protein extracts from elicited pea tissue. After 2.5 h incubation of DMDI **10** with pea crude protein extracts, the major compound produced was identified as an isoflavene, 7,2′-dihydroxy-4′,5′-methylenedioxyisoflav-3-ene (DMDIF

12), (R_t = 14.2 min) (Fig. 6b). Previous preliminary experiments had also demonstrated the production of DMDIF **12** from (−)-DMDI **10** (Dicenzo, 1998). Maackiain (R_t = 16.5 min) was also identified as a product (Fig. 6b) and confirmed to be (−)-maackiain **6** by chiral HPLC analysis. The other compounds produced could not be definitively identified. The HPLC profiles indicated that the same compounds were produced in both the absence and the presence of NADPH or FAD co-factors. A small amount of the isoflavene **12** was produced with crude proteins obtained from non-elicited tissue, but no DMDIF **12**, (−)-maackiain **6** or any of the other unknowns were produced by boiled (10 min) protein extracts of elicited tissue (Fig. 6c). Non-metabolized DMDI **10** and (−)-sophorol **5**, presumably from the non-enzymatic oxidation of DMDI **10**, and other unknowns were detected in the reaction mixture containing the boiled proteins (Fig. 6c). Preliminary tests indicated that DMDIF **12** could be metabolized further by protein extracts from pea, but the products were not identified (Kaimoyo, 2005).

3. Discussion

When RNAi constructs of IFR, SOR, and HMM were used to block the production of IFR, SOR and HMM proteins, either no (+)-pisatin **8** or reduced levels of the phytoalexin were produced in most of the transgenic hairy root lines after elicitation with $CuCl_2$. In addition, the IFRi and SORi hairy roots accumulated the two intermediates [DMD **3** and (−)-sophorol **5**, respectively] expected if IFR and SOR are involved in the biosynthesis of (+)-pisatin **8**. These results are consistent with previous results that indicated that the synthesis of (+)-pisatin **8** involves some of the same chiral intermediates that are involved in the biosynthesis of (−)-pterocarpans (DiCenzo and VanEtten, 2006; Paiva et al., 1994; Wu and VanEtten, 2004). Neither DMD **3** nor (−)-sophorol **5** was detected in elicited or non-elicited non-transgenic pea tissue, consistent with the involvement of these intermediates in a channeled pathway as lack of detection of intermediates is common for channeled pathways (Hrazdina and Jensen, 1992; Winkel, 2004).

RNAi hairy roots blocked at HMM were expected to accumulate (+)-6a-HMK **7** and to synthesize no (+)-pisatin **8**. Whereas some of the transgenic HMMi hairy root lines were deficient in (+)-pisatin **8** biosynthesis, an accumulation of (+)-6a-HMK **7** and other compounds that might have provided insight into the late steps of (+)-pisatin **8** biosynthesis, do not occur in any of the HMMi hairy root lines (Fig. 3b). During the course of the current study, another *O*-methyltransferase, hydroxyisoflavanone-4′-*O*-methyltransferase (HI4′*O*MT), that catalyzes the SAM-mediated methylation of 2,7,4′-trihydroxyisoflavanone **15** to produce 2,7-dihydroxy-4′-methoxyisoflavanone **16** (Fig. 7) was characterized in licorice (*Glycyrrhiza echinata*) (Akashi et al., 2003). The discovery of this *O*-methyltransferase resolved the mystery of how formononetin **1** is produced by legumes. The key initial steps for the biosynthesis of all isoflavonoids are summarized in Fig. 7 (Dixon, 1999; Aoki et al., 2000). It had long been thought that methylation at the 4′ position of daidzein **17** (dashed arrow, Fig. 7) was responsible for the production of formononetin **1**. However, enzymes isolated from legumes that methylated daidzein **17** always methylated the 7-hydroxy group position of daidzein **17** (7I*O*MT), rather than the 4′-hydroxy group, and produced isoformononetin **18** (Liu and Dixon, 2001, and references within). A gene for an enzyme, 2-hydroxyisoflavanone dehydratase (HID), that dehydrates the 2,7-dihydroxyisoflavones has been isolated from several legumes (Akashi et al., 2005; Hakamatsuka et al., 1998) and has provided a better understanding of the biosynthesis of both formononetin **1** and daidzein **17** by legumes (Fig. 7) as well as provided insight into our results with the HMMi hairy root lines.

There are two copies of HMM in pea and two cDNAs of HMM (HMM1 and HMM2) with deduced amino acid sequences of 96% identity have been isolated from pea tissue elicited to produce (+)-pisatin **8** (Wu et al., 1997). The *HI4′OMT* isolated from licorice shows a remarkably high sequence similarity (>80% identity) to these HMMs and Akashi et al. (2003) speculated that the HI4′*O*MT of licorice may be functionally equivalent to HMM in pea. Results of recent studies (Akashi et al., 2006) on the substrate specificities of these two HMMs support Akasihi's hypothesis and are consistent with one of the HMMs (HMM1) being HI4′*O*MT and HMM2 being the specific *O*-methyltransferase that methylates (+)-6a-HMK **7**. The licorice HI4′*O*MT and HMM1 have 20- and 4-fold higher methylation efficiencies (i.e., relative V_{max}/K_m) for 2,7,4′-trihydroxyisoflavanone **15** than for (+)-6a-HMK **7**. In contrast, HMM2 had a higher V_{max} and lower K_m on (+)-6a-HMK **7** and methylated (+)-6a-HMK **7** 67-fold more efficiently than it methylated 2,7,4′-trihydroxyisoflavanone **15**.

The two genomic copies of HMM in pea are within 18.7 kb or less of each other (Wu et al., 1997). Their close proximity is consistent with a gene duplication of *HI4′OMT,* a ubiquitous gene in legumes (Akashi et al., 2003). The HI4′*O*MT could have retained its function as in all other legumes while the duplicate could have evolved in pea for the specific HMM activity. While the deduced amino acid sequences of HMM1 and HMM2 are 96% identical, modeling of the HMM proteins has identified three or four active site residues that may be responsible for their different substrate preferences (Akashi et al., 2006). Because of their high sequence similarities, the pea RNAi constructs of HMM (HMM1 was used) would be expected to block expression of both HMM genes (Watson et al., 2005). Thus, if one of the HMM genes in pea is the equivalent to the *HI4′OMT* found in most legumes, the HMMi transgenic hairy roots of pea may not be able to synthesize pisatin **8** (or maackiain **6**) because the production of formononetin **1** needed for the synthesis of these compounds is blocked (Figs. 1 and 7). The blockage of for-

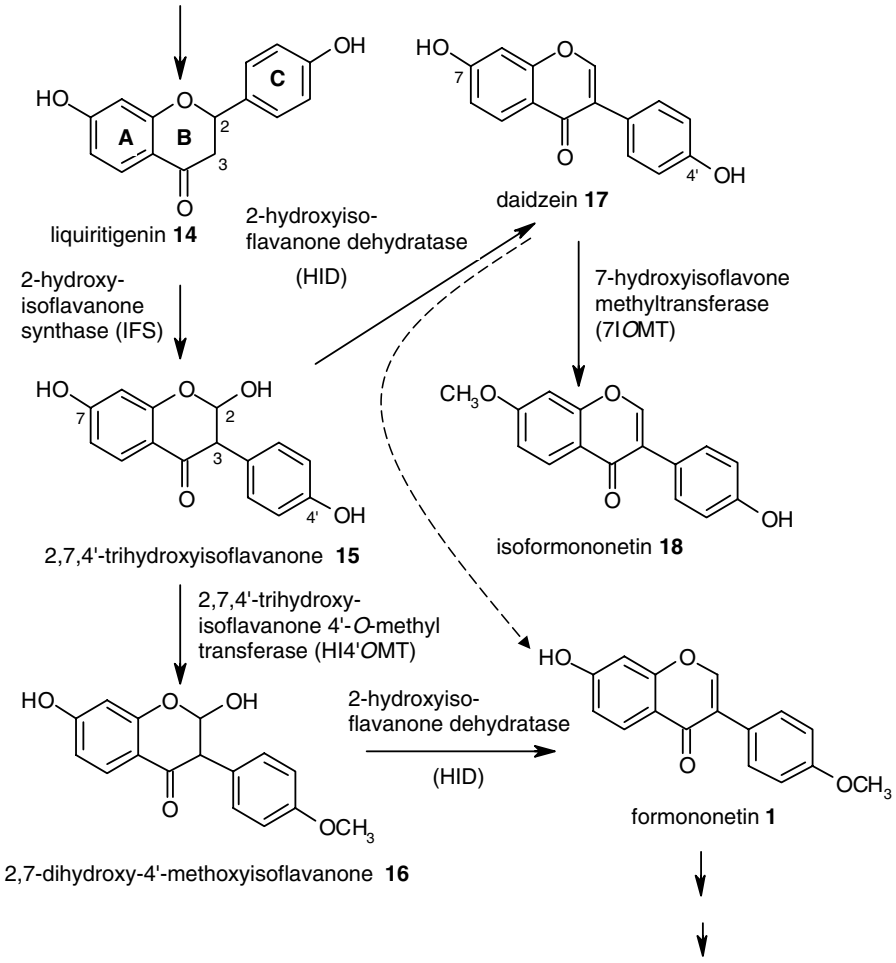

Fig. 7. Early known and hypothesized steps in the biosynthesis of isoflavonoids. The dashed arrow was the original proposed pathway by which formononetin **1** was believed to be synthesized from daidzein **17**.

mononetin **1** production could result in accumulation of isoliquiritigenin, liquiritigenin **14**, and 2,7,4′-trihydroxyisoflavanone **15** (Figs. 3b and 7). The 2,7,4′-trihydroxyisoflavanone **15** could be dehydrated by HID to produce daidzein **17**, which could subsequently be methylated by 17 7IOMT to give rise to isoformononetin **18** (Fig. 7).

The metabolism of the (−)-DMDI **10** isomer to the isoflavene DMDIF **12** (Fig. 4) by protein extracts from elicited pea tissue (Fig. 6b) is of interest in that as an intermediate, DMDIF **12** could both provide an explanation of how the configuration is reversed in the (+)-pisatin **8** biosynthetic pathway as well as for the origin of the oxygen of the C-6a hydroxy group. A recent chemical synthesis of 6a-hydroxypterocarpans has an isoflavene and a 3,4,2′ triol as intermediates **13** (van Aardt et al., 2001). The reaction sequence can serve as a model for how the stereogenic centers in (+)-pisatin **8** could be produced from the isoflavene DMDIF **12** and for how the oxygen in the 6a-hydroxy group of pisatin **8** could be derived from water as demonstrated by Matthews et al. (1987, 1989). In this scheme, an epoxidase is hypothesized to catalyze the formation of an epoxide at carbons 3 and 4 of the isoflavene, while an enantioselective epoxide hydrolase (Botes et al., 2005) would subsequently convert this epoxide to the 3,4,2′ triol **13** shown in Fig. 4. This triol would then be dehydrated by an enzyme similar to the hydroxisoflavanol dehydratase (HILD) that produces medicarpin **11** and maackiain **6** (Fig. 1). The combined effects would be the creation of a new chirality with the additional feature that the oxygen at the 6a-hydroxy position could be derived from water because of the participation of the epoxide hydrolase. Although Banks and Dewick (1983) did not find isoflavenes to be incorporated well into (+)-pisatin **8**, feeding experiments in alfalfa (Martin and Dewick, 1978, 1980) supported the participation of isoflavenes as precursors to coumestans such as coumestrol. Coumestans are structurally similar to pterocarpans but they have not been reported in pea.

4. Concluding remarks

While studies on the production of phytoalexins by all parts of the pea plant (Ingham, 1979) have consistently shown that (+)-pisatin **8** is the only phytoalexin produced in large amounts within 24–48 h after elicitation, Stoessl (1972) reported that elicited pea pods synthesized small amounts of (−)-maackiain **6**. Thus, although it has long

been known that pea has the ability to synthesize (−)-pterocarpans, the synthesis of pterocarpans with this configuration is not commonly reported. In the current study, the hairy roots that produced large amounts of (+)-pisatin **8** also produced small amounts of (−)-maackiain **6** (Fig. 3). Thus, the ability of crude protein extracts from elicited pea hairy root tissue to produce (−)-maackiain **6** from (−)-DMDI **10** is consistent with the presence of a pea HILD that produces a (−)-pterocarpan from a (−)-isoflavanol as has been found in alfalfa and chickpea plants that are synthesizing (−)-pterocarpans (Guo et al., 1994a,b). Of particular significance with respect to the synthesis of (+)-pisatin **8** in pea is the fact that the production of (−)-maackiain **6** was also greatly reduced or blocked in hairy roots that contained SORi (Fig. 3d vs. a). The blockage of both (+)-pisatin **8** and (−)-maackiain **6** production supports the hypothesis that in pea (−)-DMDI **10** is the intermediate that serves as the branch point for the two different types of configuration (Fig. 1).

5. Experimental

5.1. Microorganisms and plasmid vectors

E. coli strain DH5α was used as the host for the binary vectors and *E. coli* strain HB101, carrying the helper plasmid pRK2013 (Figurski and Helinski, 1979) was used to mobilize the binary vectors from *E. coli* DH5α to *A. rhizogenes* during triparental mating. Plasmid pRK2013 carries the *npt*II gene conferring kanamycin resistance. *A. rhizogenes* strain R1000 (nalidixic acid resistant) was a gift from Dr. M. Hawes, University of Arizona, Tucson, AZ. The binary vector, pFGC5941was a gift from Dr. R. Jorgensen, University of Arizona, Tucson, AZ: http://www.arabidopsis.org/abrc/catalog/vector_1.html. This vector is based on binary vector pCAMBIA1200. It has a cassette bearing two multiple cloning sites that facilitate the cloning of a gene in the sense and anti-sense orientation relative to the CaMV 35S promoter thus producing inverted repeats of the inserted gene. The CaMV 35S promoter drives the transcription of RNA inverted repeats. pFGC5941 also has a copy of the basta resistance (bar) gene for the screening of transgenic plants and the *npt*II gene for selection of transformed bacteria.

The fungus *Colletotrichum trifolii* isolate T456 is from a previous study (Delserone et al., 1992) and p2B1 and p3B1, two independent vectors carrying cDNA copies of SOR cloned in pBluescript vector in *E. coli* DH5α, were from previous work (DiCenzo and VanEtten, 2006.)

5.2. Chemicals

(−)-Maackiain **6** was extracted from red clover roots (VanEtten et al., 1983), (−)-sophorol **5** was produced from (−)-maackiain **6** using *C. trifoili* isolate T456 (DiCenzo and VanEtten, 2006), (+)-6a-HMK **7** was made from (+)-pisatin **8** using a strain of *Aspergillus nidulans* carrying the pisatin demethylase gene (Preisig et al., 1989), and (+)-pisatin **8** was extracted from pea tissue elicited for (+)-pisatin **8** biosynthesis with copper chloride (Sweigard et al., 1986). (−)-DMDI **10** was made from (−)-sophorol **5** by three different methods. In the first method, (−)-DMDI **10** was made as described in DiCenzo and VanEtten (2006) using an *E. coli* DH5α strain carrying a cDNA (p2B1 or p3B1) for SOR. In the second method, (−)-DMDI **10** was produced by the reduction of (−)-sophorol **5** with sodium borohydride as described by Anjaneyulu et al. (1965). (−)-Sophorol **5** (400 mg) EtOH (3 ml) were added to $NaBH_4$ (6.7 mg) and shaken at 150 rpm for 3 h at room temperature. The EtOH was evaporated to dryness under a stream of nitrogen gas and H_2O (2 ml) were added to the residue. (−)-DMDI **10** was extracted by partitioning with CH_2Cl_2 (4 ml) and purified by thin layer chromatography (TLC) or HPLC.

(−)-DMDI **10** also was routinely made by bioconversion of (−)-maackiain **6** using *C. trifolii* isolate T456. During the conversion of (−)-maackiain to (−)-sophorol **5**, the fungus also converted part of the (−)-sophorol **5** to the same isomer of (−)-DMDI **10** that is the major product made by the recombinant SOR in *E. coli*. For the routine production of (−)-DMDI **10** by this method, (−)-maackiain **6** (24 μg/ml) and 1% *C. trifolii* mycelium (w/v) in 500 ml of GA broth (Matthews et al., 1987) were incubated with shaking for 48 h at 25 °C. With all procedures, care was taken to avoid exposing (−)-sophorol **5** to basic conditions to prevent racemization.

7,2′-Dihydroxy-4′,5′-methylenedioxyisoflav-3-ene (DMDIF **12**) was made from (−)-maackiain **6** by heating (−)-maackiain **6** (2 mg) in concentrated HCl (100 μl) in EtOH (3 ml) as described by Banks and Dewick (1982a). The EtOH was dried and the resulting residue dissolved in H_2O. The isoflavene was extracted from the aqueous phase by partitioning with CH_2Cl_2 (3× ml), which was reduced to dryness under nitrogen gas, and the DMDIF **12** purified from the residue by HPLC or TLC.

Formononetin **1** and daidzein **17** were purchased from Sarsyntex (Merignac, France). Liquiritigenin **14** and isoliquiritigenin were purchased from Indofine Chemical Company (Hilsborough, NJ). Isoformononetin **18** was a gift from Dr. R.A. Dixon, Samuel Roberts Noble Foundation, Ardmore, OK and 2,7,4′-trihydroxyisoflavanone **15** was a gift from Dr. S.-I. Ayabe, Nihon University, Japan. Toluene, EtOAc, MeOH and CH_3CN were purchased from Honeywell International Inc. (Burdick and Jackson, Muskegon, MI).

5.3. Construction of RNAi binary vectors for HMM, IFR and SOR

The HMM1 and SOR cDNAs were obtained from a cDNA library constructed from RNA isolated from infected pea seedlings (DiCenzo and VanEtten, 2006; Wu et al., 1997). The pea IFR cDNA (Paiva et al., 1994) was

a gift from Dr. N. Paiva, Samuel Roberts Noble Foundation, Ardmore, OK. To construct the binary vectors for RNAi, 400-bp fragments from the 5′- or 3′-end of each cDNA of the three genes were amplified by PCR. The PCR primers were designed to include BamHI and SwaI restriction sites at their 5′ ends and XbaI and AscI sites at their 3′ ends. HMM was amplified using HMM-3HF, 5′-TCTAGAGGCGCGCCGGCACGAGTAGAAATGGA-3′ and HMM-3HR, 5′-CGGGATCCA-TTTAAATCAACAACCTGTGGCTGATC-3′. SOR was amplified with 5′-CCTCTAGAGGCGCGCCATGGCAGAGGGGAAAGG-3′ and 5′-CGGGATCCATTTAA- ATGCAACACTGAATCGC-3′. IFR was amplified with 5′-CCTCT AGAGGCGCGCCATGGCAACTGAAAAC-3′ and 5′-CGGGATCCATTTAAATCTCATACACTGCATC-3′. The PCR conditions for the amplification of each gene were similar. They consisted of an initial heat denaturation step at 95 °C for 5 min, followed by 26 cycles of 94 °C, 1 min, 58 °C, 45 s, 72 °C, 1 min, and a final 10 min cycle at 72 °C in a MJ Research PTC 100 thermal cycler (Global Medical Instrumentation, Inc., Ramsey MN, USA).

Each PCR product was divided into two portions. The first portion was digested with AscI and SwaI and cloned into the AscI–SwaI restriction endonuclease sites of the multiple cloning sites adjacent to the CaMV 35S promoter of the binary vector pFGC5941. The resulting three binary vectors contained one 400-bp fragment of the HMM, SOR or IFR gene in the sense orientation relative to the CaMV 35S promoter. The second portion of each PCR amplification product was digested with BamHI and XbaI and cloned into the XbaI–BamHI sites of the second MCS of pFGC5941. The three resulting RNAi vectors contained two copies of the 400-bp fragment of HMM, SOR or IFR arranged as an inverted repeat separated by the intron carried by pFGC5941. The binary vectors were mobilized into *A. rhizogenes* strain R1000 via the helper plasmid pRK2013 using the triparental mating method of Deblaere et al. (1987).

5.4. Production of hairy roots

Hairy roots of pea were constructed as previously reported (Wen et al., 1999) with slight modifications. Briefly, seeds of *P. sativum* L. var. Little Marvel (obtained from The Meyer Seed Company, Baltimore, MD), were surface sterilized and grown on 0.8% water agar in magenta jars under sterile conditions for 8–9 days. Short (10–12 mm) sections of stem were cut and planted up side-down on MS (Murashige and Skoog, 1962) medium semi-solidified with 2 g l^{-1} gelrite™ and supplemented with 3% sucrose and 500 mg l^{-1} carbenicillin disodium salt. Each stem was inoculated with a suspension culture (10–15 μl) of 1×10^9 CFU/ml *A. rhizogenes* R1000 carrying the pFGC5941 with the inverted repeat copies of SOR, HMM or IFR genes. *A. rhizogenes* carrying no binary vector (R1000ni) or carrying pFGC5941 without any inserted gene (R1000e) were used to generate hairy roots serving as controls. Hairy roots began to emerge after 10 days and were excised and transferred onto hormone- and herbicide-free Gamborg's B5 (Gamborg et al., 1968) semi-solid gelrite™ medium supplemented with 3% sucrose and 500 mg l^{-1} carbenicillin disodium salt. Hairy roots lacking pFGC5941 were inhibited by basta allowing the resistance to basta (2 mg l^{-1}) to be used for selection of stable and clonal transgenic hairy root cultures. The integration of the basta gene into the transformants was confirmed by RT-PCR using the oligomers BarF, 5′-TGCACCATCGTCAACCAC-3′ and BarR, 5′-ACAGCGACCACGCTGTTGAA-3′. Hairy roots were sub-cultured every 3–4 weeks onto fresh Gamborg's B5 plant tissue culture medium semi-solidified with gelrite™.

5.5. Elicitation, extraction and HPLC analyses of (+)-pisatin and other metabolites

Hairy root tissue was elicited to produce (+)-pisatin **8** and other metabolites by immersing the tissue in 0.08 mM $CuCl_2$ for 1 h at 24 °C. The tissue was removed from the $CuCl_2$ solution, transferred to a beaker, and incubated under high humidity in the dark for 24–72 h at 24 °C. In the initial screen for transgenic hairy roots deficient in (+)-pisatin **8** biosynthesis and in the time course studies, (+)-pisatin **8** was extracted by immersing the root tissue in hexanes (6 v/g) and incubating the immersed roots on a gyratory shaker at 130 RPM for 5–24 h. The hairy root tissue was removed and the hexanes evaporated under vacuum. The residue was dissolved in EtOH (0.l–1 ml). The presence of (+)-pisatin **8** in the extracts was determined by scanning for the characteristic UV absorption spectrum of (+)-pisatin **8** with a Beckman DU®-64 UV absorbance spectrophotometer (Beckman-Coulter, Fullerton, CA, USA). The pisatin concentration was determined from its absorbance at 309 nm using an extinction coefficient of $10^{3.86}$ (Cruickshank and Perrin, 1965).

To analyze transgenic hairy roots for the presence of other secondary metabolites along with (+)-pisatin **8**, hairy root tissue was ground in 4 volume of EtOH–H_2O (7:3, v/v). An additional 0.25 volume of H_2O were added to the extract and the EtOH was evaporated in vacuuo. The aqueous phase was partitioned with EtOAc (4 vol), or CH_2Cl_2 (1 vol) or $CHCl_3$ and the solvents were evaporated under vacuum. The dry residue was dissolved in EtOH (0.1–1 ml) ethanol and analyzed by HPLC.

Secondary metabolites were analyzed using an HPLC system (Waters Inc. Milford, MA, USA) consisting of two 515 pumps, a 717 plus Autosampler and a Photodiode Array Detector 996. For most analyses, the metabolites were separated using a C18 reversed phase column (4 mm × 260 mm, Alltech or Discovery) and an CH_2CN–H_2O gradient of 25–55% over a 20–60 min run at the flow rates of 0.5–1 ml/min. To separate optical isomers of pisatin **8** and maackiain **6**, a chiral HPLC column (Chrialcel OD-RH, 4.6 × 150 mm) was used with a CH_3CN–H_2O (1:1, v/v) solution run for 30 min. The HPLC data were collected via an online system and ana-

lyzed by Millennium 32™ software. Authentic standards were used to assist in the identification of the compounds. TLC analysis was done on Analtech TLC plates (UV detection at 254 nm; Uniplate, Newark, DE or EM Sciences, silica gel 60 F_{254}] Merck Kgaa, Darmstardt, Germany) with solvent systems consisting of 10:1, $CHCl_3$:MeOH or 1:1 and 60:40 toluene:EtOAc. After purification by TLC, the metabolites were analyzed for their UV absorption spectrum with a Beckman DU®-64 UV absorbance spectrophotometer.

Some metabolites were further characterized by atmospheric pressure chemical ionization (APCI) mass spectrometry and their *m/z* ratios compared to those of authentic standards. The standards were pisatin **8** (*m/z* of 314), daidzein **17** (*m/z* = 254), isoformononetin **18** (*m/z* = 268), 2,7,4′-trihydroxyisoflavanone **15** (*m/z* = 273), (−)-sophorol **5** (*m/z* = 300) and 7,2′-dihydroxy-4′,5′-methylenedioxyisoflav-3-ene **12** (*m/z* = 284).

5.6. In vitro metabolism of (−)-DMDI by protein extracts of pea

Elicited normal pea roots or hairy roots were incubated at 23 °C overnight and used immediately or frozen at −80 °C. Crude proteins were extracted from 2 g of elicited or non-elicited tissue by grinding the tissue to a fine powder in liquid N_2 and adding 4 ml Werth's micro buffer [0.4 mM EDTA, 2 mM sodium bisulfite 7 mM sodium borate, 4 mM diethyl thiocarbamic acid, 50 mM ascorbic acid, 10% (w/v) polyvinyl pyrolidone (PVP-40) and 0.17% β-mecarptoethanol; pH 7.5] (Werth, 1985). The suspension was centrifuged at 12,000 RPM for 15 min at 4 °C to obtain a supernatant containing the crude protein extract.

The crude protein extract (700 μl, 500–1200 μg) was incubated with 50 μl of 0.3 mM (−)-DMDI **10** without any co-factors or with 300 μM NADPH or FAD as co-factors at 26 °C for 5, 15, 30 min, 2.5 h or overnight. As controls, (−)-DMDI **10** was incubated with boiled (10 min) crude protein extracts. The products of the enzyme reaction were extracted by partitioning with 3 ml CH_2Cl_2 (3 ml), with the CH_2Cl_2 was removed by evaporation under nitrogen. The residue was dissolved in EtOH and analyzed by HPLC as described above.

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