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Research article

Isoflavonoids are present in *Arabidopsis thaliana* despite the absence of any homologue to known isoflavonoid synthases

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Abstract

Extracts from *Arabidopsis thaliana* leaves and inflorescence stalks and from *Lepidium sativa* (Brassicaceae) seedlings were analysed by HPLC-MS-SIM and by five isoflavonoid-specific ELISA methods after the HPLC fractionation of samples, in order to determine presence of isoflavonoids. Individual ELISAs were specific for daidzein, genistein, biochanin A and for their derivatives substituted either at the 4′- or at the 7- positions. Both analytical approaches revealed homologous spectra of isoflavonoids in both plant species. As the ononin specific immunoassay was not available this compound was only detected by HPLC-MS. Formononetin and prunetin represented the main aglycones followed by biochanin A, daidzein and genistein; sissotrin was the most abundant isoflavonoid glycoside followed by ononin, daidzin and genistin. The content of individual compounds ranged from a few micrograms up to 2.2 mg kg^{-1} (dry weight). Expression profiles of *A. thaliana* genes homologous to enzymes involved in isoflavonoid synthesis and metabolism were extracted from publicly available transcriptomic datasets for various tissues. Genes likely to be involved in important steps of the isoflavonoid metabolism in *A. thaliana* were identified. However, in accord with the previously published data, no homologue to isoflavone synthases known from the Fabaceae plants was found. These aryl migrating enzymes belong to the CYP93C family that is absent in *A. thaliana*. We conclude that another gene must be responsible for biosynthesis of the isoflavone skeleton in Brassicaceae.

Keywords: Arabidopsis thaliana; Brassicaceae; HPLC-MS; Immunoassay; Isoflavone; Isoflavone synthase; *Lepidium sativa*

1. Introduction

Due to their numerous biological activities, isoflavones (3-phenylchromones) have been drawing attention of scientists for a long time. They are involved in the interactions between a plant and its environmental partners from bacterial and fungal pathogens up to animal herbivores [6,29]. During last two decades, there has been an increasing interest in health-protecting and health-promoting effects of these compounds and in their possible use in human medicine [4].

The isoflavone synthase (IFS) gene encoding the first enzyme of the isoflavone metabolic pathway has been cloned from several leguminous species and sequenced [1,11,23,26]. Soybean IFS has been expressed in yeast and in several non-leguminous plants, which were considered to be isoflavone non-synthesizing, namely *Arabidopsis thaliana*, *Nicotiana tabacum* and *Zea mays* [11,20,30]. The IFS transgenes not only produced the expected isoflavone aglycone (i.e. genistein) but also its glycosides (namely genistin in *A. thaliana*, genistin and malonyl genistin in *N. tabacum*). This observation led the authors to the conclusion that the endogenous enzymes of non-leguminous plants were able to metabolise genistein as a novel substrate. It was, however, not taken into account, that

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HPLC-MS-SIM, high performance liquid chromatography–mass spectroscopy-SIM mode; IFS, isoflavone synthase; RT-PCR, reverse-transcription polymerase chain reaction.

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these supposed isoflavone non-producers may rather be low-level producers [20].

Reports on isoflavonoids in the Brassicaceae family have been limited to only a few notes so far. Mazur [21,22] found low levels of daidzein and genistein in two cultivars of *Brassica oleracea* during his extensive HPLC-MS screening for dietary sources of phytoestrogens; an immunochemical indication of formononetin in *Lepidium sativa* was given by Davidova [7]. Here we bring the evidence of the whole spectrum of isoflavonoids in wild type *A. thaliana* and *L. sativa*. In addition, the overview of *A. thaliana* homologues of genes encoding isoflavonoid biosynthetic enzymes in legumes is given together with their expression profiles based on microarray analyses.

2. Results

2.1. HPLC-ELISA and HPLC-MS analysis of the extracts

Water/ethanolic extracts from both, *Arabidopsis thaliana* and *Lepidium sativa*, were positively tested for several types of isoflavonoid-like immunoreactivity. After HPLC fractionation, the retention times of the immunoreactive entities corresponded to those of authentic standards (Fig. 1). Two independent immunoassays were available for daidzein (lines 1 and 2) and another two for biochanin A (lines 3 and 5). In both cases, the results of parallel assays corresponded satisfactorily. The HPLC-MS-SIM analysis confirmed the presence of all immunoreactive isoflavones and moreover, ononin, for which an immunoassay was not available, was detected (Table 1). Representative chromatograms of *A. thaliana* extract for selected ions are given in Fig. 2. The spectrum of isoflavones was similar in both plants, with the estimated content of individual substances ranging from a few micrograms to about 2.2 mg kg^{-1} (dry weight). The aglycones as well as the 7-*O*-glycosides were present in all samples, and the 4′-methoxy isoflavones were more abundant than 4′-hydroxy isoflavones (Table 2).

2.2. Searching in A. thaliana genome

Protein sequences of genes involved in the isoflavonoid synthesis and metabolism (chalcone synthase, chalcone-flavanone isomerase, chalcone reductase, isoflavone synthase, isoflavone reductase, isoflavone 2′-hydroxylase, isoflavone 7-*O*-methyltransferase) downloaded from GenBank were used for the homology-based search using WU-BLAST2 on TAIR. 51 candidate genes forming eight gene families were identified (Table 2). Publicly available Affymetrix ATH1 microarray-based transcriptomic datasets [5] were used to determine spatial and temporal expression patterns of candidate genes. Expression profiling analyses led to the identification of several genes likely to be involved in the isoflavone synthesis and metabolism in *Arabidopsis*. Out of the 51 candidates, 46 (90%) genes were present on the ATH1 genome array. For these genes, expression profiles in individual organs and tissues were obtained (Table 3). In each gene family, there was at least one

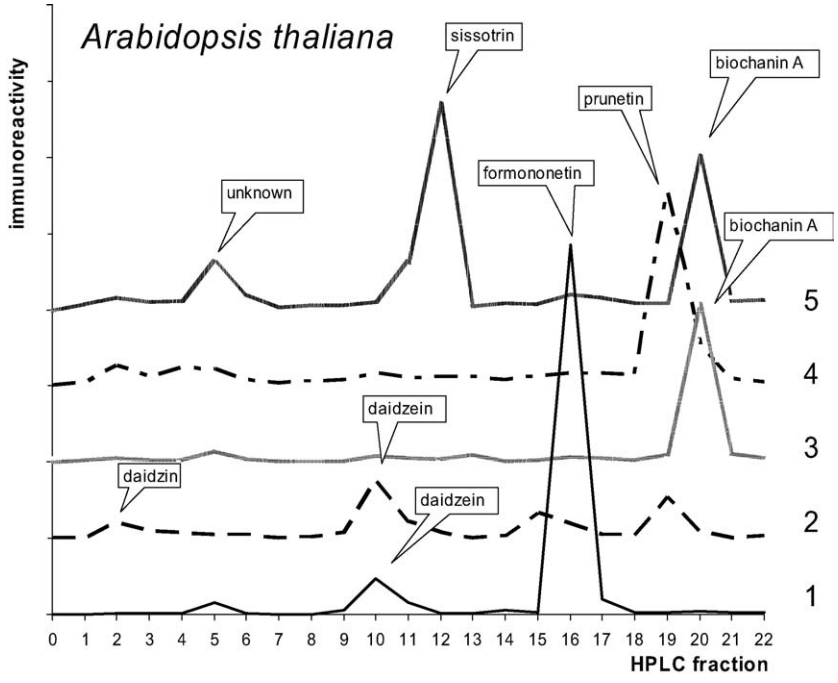

Fig. 1. Isoflavonoid immunoreactivity in HPLC fractions of *A. thaliana* leaves.
Extract from 12.5 mg of *A. thaliana* leaves was chromatographed, the fractions collected, dried and reconstituted in the assay buffer for immunoanalysis. Five ELISAs highly specific to individual isoflavonoid subgroups were used. One segment on the Y axis corresponds to 0.1 mg kg^{-1} of a respective substance in dry matter.
Column: Purospher Star RP18e 125/4 column with a guard column Purospher Star RP18e 4/4 (Merck, Germany). Gradient elution: A (0.05% acetic acid in water) and B (methanol) as followed (all steps linearly): 0 min, A = 60%, B = 40%; 5 min, B = 52%; 20 min, B = 70%; 25 min, B = 100%; 25–45 min, B = 100; then step to A = 60% and reconditioning of the column for 10 min. Flow rate was 0.8 ml min^{-1} and the temperature was set at 25 °C. 1.0 ml fractions were collected.