ORGAN SPECIFIC PRODUCTION OF (LONG-CHAIN) GLUCOSINOLATES IN THE CRUCIFERAE SPECIES *CAPSELLA BURSA-PASTORIS* (SHEPHERD'S PURSE)

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INTRODUCTION

Wild Cruciferae species are potentially useful sources of desirable genetic traits that might be transferred to oilseed *Brassica*. In *Capsella bursa-pastoris* (shepherd's purse) resistance to flea beetles (*Phyllotreta cruciferae*) (Feeny *et al.*, 1970, Pivnick, pers. comm.) and blackspot disease (*Alternaria brassicae*) have been observed (Conn *et al.*, 1988).

The glucosinolate (GS) content of shepherd's purse was of interest for two reasons. First, although very controversial, GS or their derivatives (degradation products/phytoalexins) play putative roles in determining susceptibility/resistance to pests/pathogens (Chew, 1988). Second, GS profiles could possibly be used as a finger printing tool in the characterization of somatic hybrids of shepherd's purse and *Brassica*.

Preliminary analyses of shepherd's purse showed that GS were absent from cell suspension cultures, but also from green tissue of protoplast regenerants grown *in vitro*. Absence from cell cultures was not deemed unusual as GS production is commonly lost during subculturing of *Brassica* (GrootWassink *et al.*, 1990b). However, we had not previously encountered any green tissue of crucifers devoid of GS. This apparent abnormality prompted more detailed study of organs of field and growthroom plants, including feeding of GS precursors to establish expression of enzymes for main group formation (GrootWassink *et al.*, 1987). Furthermore, identities of long-chain GS were established.

MATERIALS and METHODS

Metabolite analysis. Vegetative tissues and seeds were extracted in boiling water, filtered and the filtrate applied to DEAE-Sephadex A-25 columns. The columns were washed twice with water and loaded with purified arylsulfatase (Type H-1, Sigma). The sulfatase-loaded columns were kept overnight at room temperature and the desulfated metabolites eluted with water. All enzyme activities in the eluate were inactivated by heating in a boiling water bath. 2-Nitrophenyl-ß-D-galactoside (ONPG) was added as internal standard. The eluate was subjected to HPLC separation on a reversed-phase column (Whatman C18 PartiSphere 5µm particle size) using an acetonitrile in water gradient of 1.25-22.5% (Sang and Truscott, 1984). Desulfated constituents were detected by UV absorption at 226 nm. Structural identities were established by co-elution with authentic compounds and mass spectrometry.

Structure identification. Mass spectrometry using a VG 70-250 SEQ hybrid mass spectrometer was applied to a mixture of desulfated compounds and a mixture of isothiocyanates (fast ion bombardment secondary ion mass spectrometry [FIB/MS]), to desulfated compounds separated by HPLC (plasmaspray liquid chromatography/mass spectrometry [LC/MS]) and to isothiocyanates separated by gas chromatography (electron impact mass spectrometry [GC/MS]). Isothiocyanates were generated from GS by incubation of seed extracts with thioglucosidase (myrosinase, Sigma). Pre- and postextraction of the reaction mixtures with methylene chloride yielded clean isothiocyanate solutions for GC and mass spectrometry. The GC conditions were: DB-5 fused silica capillary column (0.32 mm ID x 30 m); injector temperature 180°C; column temperature programmed from 40°C to 90°C in 45 sec, then to 250°C in 20 min.

RESULTS and DISCUSSION

GS distribution. The GS composition of various organs of shepherd's purse was determined. Seeds collected throughout the summer of 1990 from a number of Saskatoon field locations, and seeds produced in a growth room consistently contained two major GS (Fig. 1A, compound 4 and 5; see below for identification). The same GS also occurred consistently in roots, along with greatly varying amounts of two others (Fig. 1D, compound 2 and 3). The green portion of the plant can be divided into the inflorescence and the rosette leaves, for the purpose of GS content. The inflorescence, comprising the stem, small leaves and pods can each contain allylGS (Fig. 1B, compound 1) and the four unknown GS (compound 2, 3, 4 and 5), all in amounts from undetectable to predominant depending on strain, growth conditions and stage of development. The rosette leaves, constituting the only green part until bolting, contained no detectable GS at any stage of the life cycle of field and growthroom plants (Fig. 1C). This absence of GS from a vegetative part represented an exception among the Cruciferae species we had examined earlier, i.e. several *Brassica* spp, *Arabidopsis thaliana*, *Thlaspi arvense* (stinkweed) and *Descurania sophia* (flixweed).

Expression of GS pathway enzymes. To confirm whether absence of GS from the rosette leaves was related to the inability to synthesize GS, the precursor 2-nitrobenzaldoxime was fed to whole plants and to leaf and stem cuttings. All organs normally containing GS were also capable of converting 2-nitrobenzaldoxime to the corresponding 2-nitrophenylGS (Fig. 2A and C, compound 6). During root feeding, the precursor was translocated to the rosette leaves, but failed to be converted to the GS (Fig. 2B). Only the commonly observed sulfatoglucoside conjugation products were formed (GrootWassink *et al.*, 1990a). The same results were obtained with direct feeding of leaf cuttings. Cell suspension cultures of shepherd's purse also did not convert 2-nitrobenzaldoxime, while *Brassica*, *Arabidopsis* and stinkweed cultures all have this capability. To ascertain that the GS biosynthetic pathway was not interrupted in the rosette leaves, the last step in the pathway was checked by feeding desulfoallylGS. In contrast to the control system *Brassica campestris* R-500 leaves, normally devoid of allylGS, rosette leaves of shepherd's purse were unable to form allylGS from its immediate precursor. Therefore, it appears that the genes encoding GS pathway enzymes are under developmental control, i.e. repressed in rosette leaves, while constitutively expressed in all other organs of shepherd's purse. Understanding this tissue (organ) specific gene regulation might contribute to our genetic engineering efforts to eliminate GS production completely in oilseed *Brassica*.

Identification of long-chain GS. Above HPLC analyses revealed the presence of four unknown GS in shepherd's purse (Fig. 1, compound 2, 3, 4 and 5) . Structures of compound 4 and 5 were elucidated using MS. Plasmaspray LC/MS of a desulfated seed extract showed two major components with protonated molecular ions of m/z 428 and 442, respectively (Fig. 3). FIB mass spectrometry of a mixture of the desulfated compounds from seeds confirmed the two major protonated molecular ions, representing molecular weights of 427 and 441, respectively. If the mass of 238 for the S-glucosyl thiohydroximate main group was deducted, a mass of 189 and 203 remained for the respective side chains. GC/MS of the corresponding isothiocyanates (RNCS, where R is the sidechain) indicated that the two major compounds contained an aliphatic chain of 9 and 10 carbons with one double bond, respectively (Fig. 4). However, the molecular weights of these side chains were not compatible with those established by LC/MS and FIB/MS of the desulfoGS. Compatible molecular ions of 248 and 262 were obtained only if the GC separation was omitted, and mixtures of the isothiocyanates were entered directly into the mass spectrometer (FIB/MS, Fig. 5). The spectra also indicated the presence of sulfur in the side

chain of both compounds. Daughter spectra (MS/MS) of the FIB generated isothiocyanate protonated molecular ions showed a loss of 64, which was postulated to represent CH_3SOH. Heat instability of this group had apparently caused its elimination in the injector of the GC. Together with all other mass spectral evidence, it was concluded that the unknown glucosinolates contained methylsulfinyl C9 and C10 side chains. Although these long-chain GS appear rare, lower homologs occur in many species. Comparison of the LC/MS spectra of the shepherd's purse desulfoGS with those of lower homologs of *Arabidopsis* indicated the same general structure (Hogge *et al.*, 1988).

The GS identified above had each been isolated before from different crucifer species; 9-methylsulfinylnonyl glucosinolate (glucoarabin) from the seeds of *Arabis alpina* and 10-methylsulfinyldecyl glucosinolate (glucocamelinin) from the seeds of *Camelina sativa* (false flax) (Kjaer, 1960).

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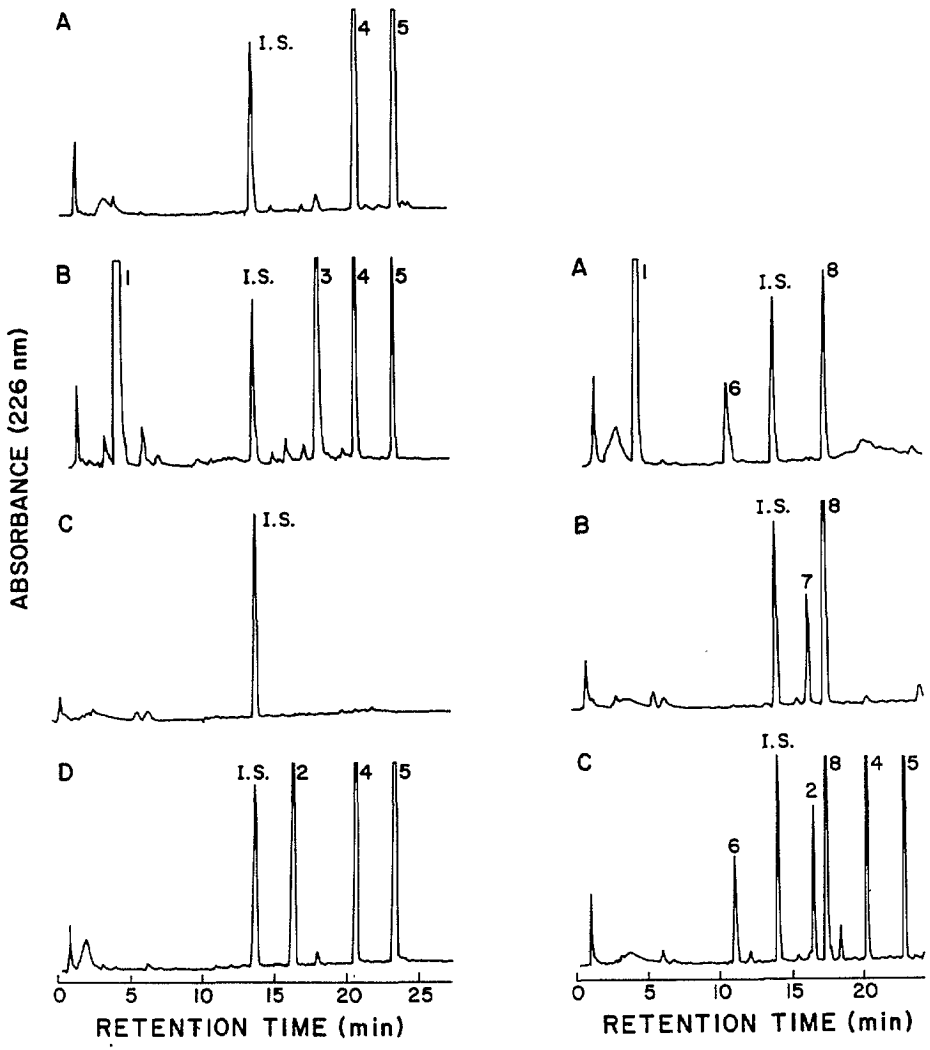

Fig. 1. HPLC analyses of desulfoglucosinolates from shepherd's purse organs. A. Seeds; B. Inflorescence (stems, small leaves, pods); C. Rosette leaves; D. Roots. Peak identification: 1. Allylglucosinolate; I.S. Internal standard (ONPG); 2, 3, 4, 5. Unknown.

Fig. 2. HPLC analyses of desulfated metabolites from shepherd's purse organs fed 2-nitrobenzaldoxime. A. Upper inflorescence stems, pods removed before extraction. Inflorescence cuttings were fed 2 days with 1mM 2-nitrobenzaldoxime in water. B. Rosette leaves, taken from soil-grown plants fed 14 days with 1 mM 2-nitrobenzaldoxime in water. C. Roots, taken from the same plants as in B. Peak identification: 1-5. See legend of Fig.1; 7. (*Z*)-2-nitrobenzaldoxime-ß-D-glucopyranoside; 8. (*E*)-2-nitrobenzaldoxime-ß-D-glucopyranoside.

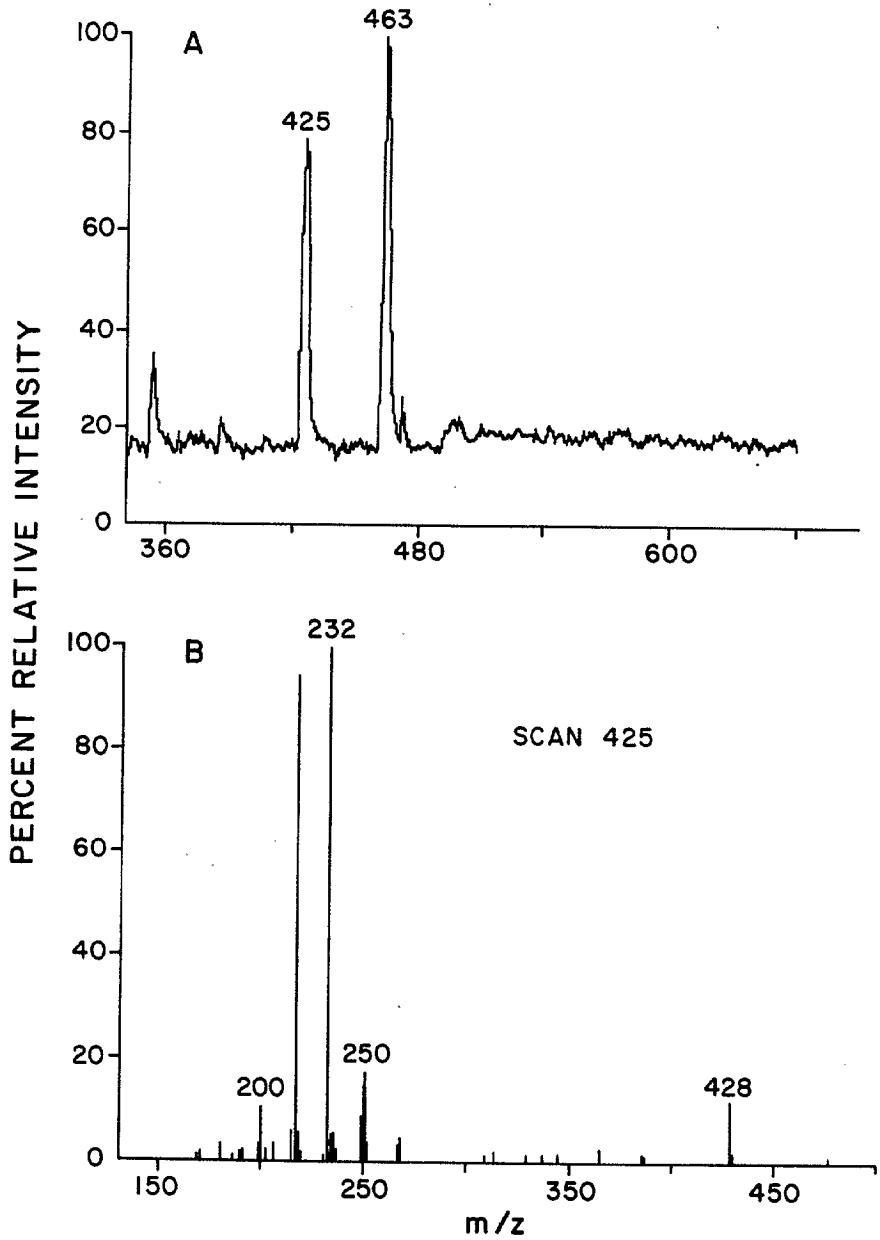

Fig. 3. LC/MS of desulfated shepherd's purse seed extract. A. HPLC reconstructed total ion chromatogram. Scan number 425 and 463 corresponded to peak 4 and 5 of Fig. 1. B. MS spectrum of scan 425.

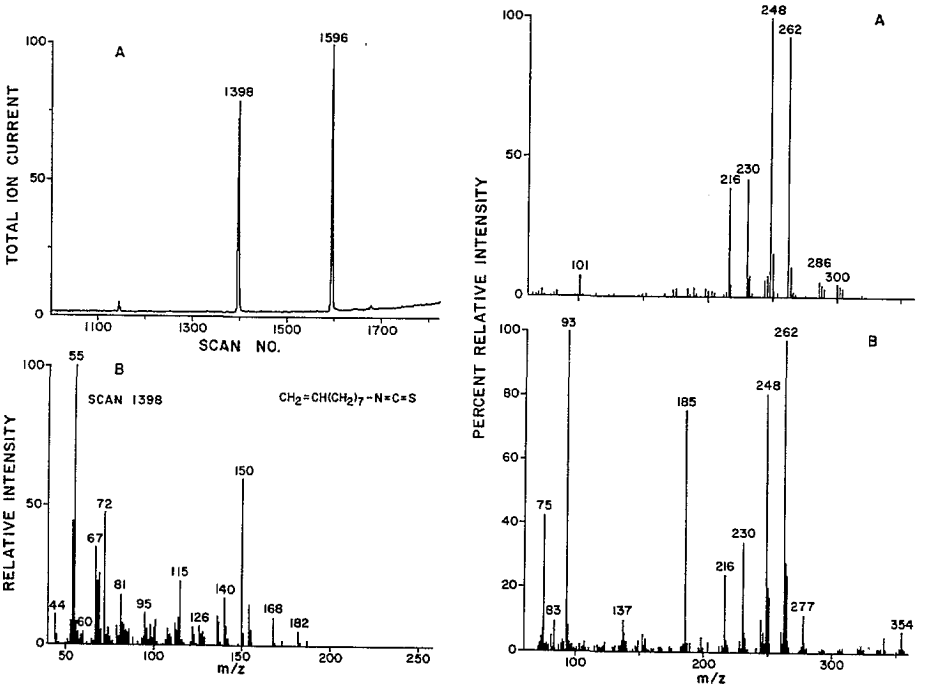

Fig. 4. GC/MS of isothiocyanates prepared from shepherd's purse seed extract. A. GC reconstructed total ion chromatogram. Scan number 1398 and 1596 corresponded to the desulfoglucosinolate 4 and 5 of Fig. 1. B. MS spectrum of scan 1398.

Fig. 5. MS spectra of isothiocyanate mixtures prepared from shepherd's purse seed extract. A. Direct chemical ionization (ammonia) detection. B. FIB (+ion) detection.