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Research Article

On the distribution of phytoecdysteroids in plants

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Abstract. The occurrence and levels of phytoecdysteroids in the seeds and other parts of plants grown from the seeds of 180 randomly selected plant species were assessed and compared. Ecdysteroids are frequently detectable in leaves and flowers, but less so in stems, roots and seeds. The seeds of 290 species were assessed for the presence of hydrolysable ecdysteroid conjugates. Low levels of conjugates were detected in a significant number of species, large amounts being present only when levels of free ecdysteroids were high. Individual plants of *Arabidopsis thaliana* were assessed for the presence of phytoecdysteroids. While plants of this species are generally ecdysteroid negative, individual plants in the population accumulate low levels of ecdysteroids. Extracts of seeds of 50 "ecdysteroid-negative" species were concentrated and partially purified to determine if they possess ecdysteroids at levels below the usual detection levels. Ecdysteroids were detectable by radioimmunoassay in almost all of these concentrated samples. Thus, all lines of evidence point to the conclusion that all species of plants have the capacity to produce at least low levels of phytoecdysteroids. This has important implications for the protection of crop species through enhancing ecdysteroid levels by breeding/genetic modification strategies.

Key words. *Arabidopsis*; chemotaxonomy; ecdysteroid; 20-hydroxyecdysone; insect-plant relationships.

Ecdysteroids are the steroid hormones of arthropods and probably of many other invertebrate phyla. In insects, they regulate moulting and metamorphosis and may also have roles in reproduction, embryogenesis and diapause [1]. Phytoecdysteroids are analogues of these invertebrate steroid hormones (zooecdysteroids) that occur in a wide variety of plant species [2]. More than 200 analogues have been isolated to date [3]. Their presumed function is to contribute to the deterrence of invertebrate predators; evidence is gradually accumulating that they are effective toxins or antifeedants towards non-adapted insect species. However, some polyphagous species are remarkably tolerant towards ingested phytoecdysteroids [reviewed in ref. 4]. Phytoecdysteroids are apparently non-toxic to mammals and may even have a number of beneficial pharmacological and medicinal applications [5, 6]. Consequently, elevation of ecdysteroid levels in crop species may provide an effective control method for many invertebrate predators without increasing risks for human or other vertebrate consumers. Most crop species do not contain detectable levels of ecdysteroids, spinach (*Spinacia oleracea*) and quinoa (*Chenopodium quinoa*) being notable exceptions [7–10]. Elevation of ecdysteroid levels in crop species could be brought about by genetic engineering of the ecdysteroid biosynthetic pathway or its regulation to provide adequate levels of particular phytoecdysteroid analogues which are not readily detoxified even by polyphagous predators. Some evidence suggests that most, if not all, plant species retain the genetic capacity to produce ecdysteroids: the almost erratic distribution of ecdysteroid-positive species in the plant world and the geographical variation in ecdysteroid levels in

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some species. Consequently, at a time of considerable public sensitivity and resistance towards genetic engineering, identifying varieties with elevated ecdysteroid levels may be possible through traditional selection procedures. Progress in this area is hampered by our limited knowledge of the biochemistry and biology of phytoecdysteroids, with only a restricted understanding of the biosynthetic pathway(s) in plants and no knowledge of the regulation of the activity of these pathways [6, 11]. A number of facets of the distribution of phytoecdysteroids within the plant world are enigmatic. Five to six percent of all higher plant species accumulate detectable levels of ecdysteroids [12, 13], but levels vary enormously between species and the distribution of ecdysteroid-containing species is only partly understood [14, 15]. When levels in ecdysteroid-containing species are compared, distributions within the plants vary in species- and developmentally dependent ways. Ecdysteroid profiles also vary considerably, although 20-hydroxyecdysone (fig. 1) and polypodine B (5β, 20-dihydroxyecdysone) are the most common major ecdysteroids. The other analogues (usually occurring as minor components) differ from 20-hydroxyecdysone mainly in the number and position of oxygen-containing functional groups or in the nature or positions of attachment of conjugating moieties (e.g. glycosides, acyl esters) [16]. Phytoecdysteroids thus provide a rich diversity of analogues suitable for quantitative structure-activity studies in insect systems [e.g. ref. 17]. Most methods which have been applied to the analysis of phytoecdysteroids are effective in detecting a subset of the plethora of possible analogues, but may completely miss others. Generally, approaches have either used physicochemical properties of the molecule (the characteristic UV absorbance at 242 nm in alcohol which is common to most, but not all, ecdysteroids) or the biological activity based on a bioassay (e.g. the *Chilo* dipping test [12]). We have developed an approach which combines assessment of both structural similarity to ecdysone [using radioimmunoassay RIA] and biological activity (B_{II} bioassay for ecdysteroid agonist activity) [13, 18]. These methods are sensitive and robust, allowing assessment of phytoecdysteroid levels in small samples of plant material after simple solvent extraction and work-up. In this report, we present data from four experiments designed to shed light on the occurrence and distribution of phytoecdysteroids. In the first experiment, the phytoecdysteroid levels in seeds of 180 randomly chosen species were compared to the levels present in the various parts of plants grown from those seeds. In the second experiment, extracts of seeds of 290 species were examined for the presence of ecdysteroid conjugates, and in the third and fourth experiments, we considered the question of whether all species are capable of producing ecdysteroids, the levels in most species, though, being below usual detection levels.

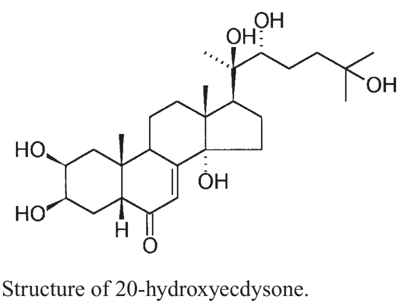

Figure 1. Structure of 20-hydroxyecdysone.

Materials and methods

Plant material

Plants of *Arabidopsis thaliana* (ecotype Col-0) were grown in a compost mix of equal parts of vermiculite, perlite, John Innes No. 2 and silver sand. Individual rosette leaves were harvested when the plants were 6 weeks old. Seeds of other species were obtained from the Seed Guild (Lanark, UK). Plants were grown in the glasshouses of the University of Exeter. After harvesting, plants were cut into the relevant sections, fresh weights were determined and the material was then freeze-dried for 4 days prior to extraction.

Extraction procedure

Seeds and freeze-dried plant samples were crushed in a pestle and mortar and a portion (25 mg) was extracted as described elsewhere [13]. Briefly, the sample was extracted with methanol (3 × 1 ml, each for 3 h at 55 °C). The pooled extracts were mixed with water (1.3 ml) and partitioned against hexane (2 ml). The aqueous methanol phase (4.3 ml) was used for ecdysteroid analysis.

Phytoecdysteroid analysis and quantification

Details of the ecdysteroid-specific radioimmunoassays and the B_{II} bioassay are given elsewhere [13, 18–20]. The two antisera used differ in their specificities for ecdysteroids. The relative binding affinities for ecdysone, 20-hydroxyecdysone and polypodine B for the DBL-1 and Black antisera are 1:1.3:10.8 and 1:68.2:124, respectively [13]. Portions of the aqueous methanol extracts were assessed by RIA with the DBL-1 antiserum (2 × 5 µl extract) and Black antiserum (2 × 10 µl extract) and in the agonist and antagonist versions of the B_{II} bioassay (4 × 20 µl). When a positive response was obtained in initial RIA, the level of RIA-positive material (in µg ecdysone equivalents/g) was quantified by repeating the RIA with five known volumes (in duplicate) of the extract, plotting RIA response against volume and determining the gradient of the regression line. Ecdysteroid-containing extracts will give well-fitted straight lines and this procedure allows the recognition of extracts giving non-linear responses and deviations arising from interference at larger sample sizes. In the case of the bioassay, a response (agonistic, antagonistic or cytotoxic) in the initial bioassay was followed up by reassessing the extract in a series of ten-fold dilutions until no further activity was seen. The threshold for detection is 344 ng ecdysone equivalents/g with the DBL-1 antiserum, 688 ng ecdysone equivalents/g with the Black antiserum and 1 µg 20-hydroxyecdysone equivalents/g in the agonist version of the B_{II} bioassay. To test for the presence of hydrolysable ecdysteroid conjugates [18, 21], portions (2 × 100 µl) of each aqueous methanol phase were evaporated in Eppendorf vials. To one portion (control), 100 µl 0.1 M sodium acetate buffer (pH 5.4) was added. To the other portion (hydrolysate), *Helix pomatia* hydrolases (Sigma, Type H1; 10 mg/ml sodium acetate buffer) were added. After 48 h at 37 °C, 0.8 ml ethanol was added and, after storing at 6 °C for several hours, the precipitated protein was removed by centrifugation (13,000 × g). The supernatant was used to quantify ecdysteroids by RIA (using the DBL-1 antiserum).

Ecdysteroids in individual plants of *A. thaliana*

Single leaves were removed from the rosettes of 334 individual (pre-flowering stage) plants of *A. thaliana* and each was placed in a 2 ml screw-top Eppendorf vial and freeze-dried for 4 days. Methanol (1 ml) was added to each vial and the leaves were extracted for 3 × 3 h at 55 °C. Aliquots (10 µl) of each extract were assessed in duplicate by RIA (with the DBL-1 antiserum). Extracts giving a positive response were reassessed at five aliquot sizes in duplicate.

Further purification of 'ecdysteroid-negative' extracts

For 50 of the seed extracts from species which were ecdysteroid-negative (listed in table 3), a portion (2.5 ml from 4.3 ml) of the 70% aqueous methanol phase was diluted to 10% methanol in water and applied to a pre-equilibrated C_{18} SPE column (Waters/Millipore, Watford, UK). The column was washed with 25% methanol in water (5 ml) and any ecdysteroids eluted with 75% methanol in water (5 ml). The cartridge was finally eluted with methanol (5 ml). The 75% methanol fraction was dried down and redissolved in 250 µl methanol and 5, 10, 25 and 50 µl aliquots were taken in duplicate for RIA. Thus, the extract was concentrated ten-fold and the maximum volume taken for RIA (DBL-1 antiserum) was increased ten-fold over the usual assay, thus increasing the sensitivity 100-fold and reducing the detection limit to 3.4 ng ecdysone equivalents/g. New glassware and plasticware were used throughout the above experimental procedure to obviate the possibility of contamination. The residues from solvents used in these procedures were all RIA-negative.

Results and Discussion

Randomness of the plant species

The seeds from which the plants were grown were provided from the catalogue of the seed supplier without selection. In total, the seeds of 408 species were sown, of which 180 germinated within 1 year. This is described as a random selection of species, but we recognise that it is subject to some bias towards species which are horticulturally favoured and to those with shorter germination times in a glasshouse environment.

Distribution of ecdysteroids in plants

Table 1 presents the data obtained by RIA with the DBL-1 and Black antisera and the agonist version of the B_{II} bioassay. However, each sample was also assessed in the antagonist version of the B_{II} bioassay. A few species showed antagonistic activity, but since all the pure phytoecdysteroids which have been tested in the B_{II} bioassay (over 100) [17, 19, 22] are agonistic, antagonistic activities will not be discussed further here (this information is available from the first-named author on request). Generally, there is excellent agreement between the data obtained with the Black and DBL-1 antisera and the agonist bioassay, taking into account the lower sensitivities of the RIA with the Black antiserum and the B_{II} bioassay.

RIA-positive material was detected in 16 of the seed extracts (8.9%), of which 4 (2.2%) had levels above 1 µg ecdysone eq./g with either the DBL-1 or Black antisera. These percentages are similar to those found earlier for a larger number of randomly selected seed extracts using the same methodology [13]. Many more leaf extracts of the species were found to be RIA positive (48%), but only leaf extracts of the 4 species for which the seeds were highly positive (*Eryngium maritimum*, *Helleborus foetidissima*, *Limonium ramosissimum*, *Silene pusilla*) contained levels above 1 µg ecdysone eq/g. Thus, the leaves of many species appear to contain detectable ecdysteroid levels. The proportions of species with RIA-positive material in the stems (16%), roots (17%) and flowers (33%) were intermediate between the seeds and leaves. Within ecdysteroid-containing species the highest levels may be present in the roots (*E. maritimum*, *Incarvillea forestii*, *L. ramosissimum*, *S. pusilla*), stems (*H. foetidissima*, *Pittosporum tenuifolium*) or seeds (*Hieracium murorum*). We and others [14, 15] have used seed extracts to assess plant species for the occurrence of ecdysteroids. However, the levels in seeds may be low in comparison to other parts of the plant (*E. maritimum*, *H. foetidissima*, *S. pusilla*) or much higher than in other parts of the plant (*H. murorum*). Thus, one must conclude that there is no consistent pattern in the distribution of ecdysteroids within plants, unlike, for example, the distribution of withanolides in solanaceous species which predominantly occur in leaves [23]. However, it can be

Table 1. Distribution of ecdysteroids (determined by RIA and B_{II} bioassay) in randomly selected plant species.

Species[a]	Family[b]	Seeds	Ecdysteroid content (RIA [DBL-1]/RIA [BL]/agonist bioassay)[c]					
			leaves	stems	roots	flowers	bulb	fruit
Acacia melanoxylon	Leguminosae-M.[a] (D)	-/-/-	-/-/C–	-/-/C–	-/-/C–			
Acaena fissistipula	Rosaceae (D)	-/-/-	-/-/C-	-/-/-	-/-/-			
Agastache foeniculum	Labiatae (D)	-/-/-	0.47/0.17/C–	-/-/-	-/-/-	0.28/-/-		
Agrimonia striata	Rosaceae (D)	-/-/-	-/0.27/-		-/-/-			
A. gyrosepala		-/-/-	-/-/C–		-/-/C–			
Alcea pallida	Malvaceae (D)	-/-/-	-/0.07/C–	-/-/-	-/0.08/-			
Allium albidum	Alliaceae (M)	-/-/-	-/-/C–		-/-/-		-/-/-	
A. carinatum ssp. *pulchellum*		-/-/-	-/-/-		-/-/-			
A. montanum		-/-/-	-/-/-		-/-/-		-/-/-	
Alstroemeria ligtu	Alstroemeriaceae (M)	-/-/-	-/0.27/C–	-/-/C–				
Amelanchier florida	Rosaceae (D)	-/-/-	-/0.13/C–	-/-/-		-/-/-		
Anagallis foemina	Primulaceae (D)	-/-/-	-/-/C–	-/-/C–		-/-/C–		-/-/C–
Androsace septemtrionalis	Primulaceae (D)	-/-/-	-/0.46/C–		-/0.27/C–			
Anthemis punctata	Compositae (D)	-/-/-	-/0.29/C–	-/-/C–	-/-/-	-/0.21/C–		
Aquilegia atrata	Ranunculaceae (D)	-/-/-	-/0.31/-		-/0.27/C–			
A. canadensis		-/0.05/C–	-/-/C–		-/-/-			
A. flavescens		-/-/C–	-/-/-		-/-/-			
Arabis (*Cardaminopsis*) *arenosa*	Cruciferae (D)	-/-/-	-/-/C–	-/-/-	-/-/-	-/-/C–		
A. drummondii		-/-/-	-/-/-	-/-/-	-/-/-			-/-/C–
A. soyeri		-/-/-	-/0.48/C–		-/-/-			
Argemone mexicana	Papaveraceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-	-/-/-		-/-/-
Arum maculatum	Araceae (M)	-/-/-			-/-/-	-/-/-	-/-/-	
Asarina barclaiana	Scrophulariaceae (D)	-/-/-	0.24/0.39/-	0.28/-/-	-/-/-			
A. erubescens		-/0.19/-	-/-/C–	-/-/-	-/-/-			
Asclepias speciosa	Asclepiadaceae (D)	-/-/-	-/0.30/C–	-/-/C–	-/-/-			
Bellevalia romana	Hyacinthaceae (M)	-/-/-	0.36/-/C–		-/-/-			
Betula globispica	Betulaceae (D)	-/-/-	-/0.17/C–	-/-/-	-/-/-			
Bromus interruptis	Gramineae (M)	-/-/-	-/-/-		-/-/-			
Buddleja globosa	Buddlejaceae (D)	-/-/-	-/-/C–	-/-/C–	-/-/-			
Buglossoides arvensis	Boraginaceae (D)	-/0.12/-	-/-/C–	-/-/-	-/-/-			
Caladrina grandiflora	Portulaccaceae (D)	-/-/-	-/-/C–	-/-/C–	-/-/C–			
Callistemon pityoides	Myrtaceae (D)	-/-/C–	-/-/C–	-/-/-	-/-/-			
Carex baccans	Cyperaceae (M)	-/0.14/-	0.15/0.27/C–		-/-/-			
C. berggrenii		-/-/-	0.21/-/C–		-/-/-			
C. flagellifera		-/-/-	-/-/C–		-/-/-			
C. secta		-/-/-	-/-/-		-/-/-			
Cerastrium boissieri	Caryophyllaceae (D)	-/-/-	-/-/-	-/-/-	-/-/-			
Chaenorhinum minus	Scrophulariaceae (D)	-/-/-	-/0.30/C–	-/-/C–	-/-/-	-/-/C–		
Chrysanthemum coronarium	Compositae (D)	-/-/-	-/-/C–		-/-/C–	-/-/C–		
Cistus albidus	Cistaceae (D)	-/-/-	0.28/0.23/C–	0.27/-/-	-/-/-			
C. hirsutus		-/-/-	-/0.28/C–	-/0.19/C–	-/-/C–			
C. ladanifer		-/-/-	-/-/C–	-/-/-	-/-/-			
C. populifolius		-/-/-	0.25/-/C–	-/-/-	-/-/-			
C. psilosepalus		-/-/-	-/-/C–	-/-/-	-/-/-			
Coleus (*Plectranthus*) *frederici*	Labiatae (D)	-/-/-	-/0.09/C–	-/-/-	-/-/-			
Consolida ambigua	Ranunculaceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-	-/-/-		
Coreopsis major	Compositae (D)	-/-/-	-/-/-	-/-/-	-/-/C–	-/-/CC–		
Coriaria microphylla	Coriariaceae (D)	-/-/-	0.22/-/C–		-/0.34/C–			
Cotoneaster amoenus	Rosaceae (D)	-/-/-	-/-/-	-/-/-	-/-/-			
C. frigidus		-/-/-	-/-/-	-/-/-	-/-/-			
C. marguandii		-/-/-	-/-/-	-/-/-	-/-/-			
Cryptostegia grandiflora	Asclepiadaceae (D)	-/-/-	-/0.08/CC–	-/-/C–	-/-/CC–			
Cyclamen hederifolium	Primulaceae (D)	-/-/-	-/0.08/C–			-/-/C–		
C. persicum		-/-/-	-/-/CC–	-/-/C–		-/-/C–	-/-/C–	
Cynoglossum hungaricum	Boraginaceae (D)	-/-/-	-/0.25/C–		-/-/-			
Deschampsia parviflora	Gramineae (M)	-/0.02/-	-/0.17/-		-/-/-			
Desmodium elegans	Leguminosae-P. (D)	-/-/-	-/0.52/C–	-/-/-	-/-/-			
Dianthus amurensis	Caryophyllaceae (D)	-/-/-	-/0.20/C–	-/-/-	nl/-/C–	nl/0.12/C–		
Draba languinosa	Cruciferae (D)	-/-/-	-/-/C–		-/-/-			

Table 1. (continued)

Species[a]	Family[b]	Seeds	Ecdysteroid content (RIA [DBL-1]/RIA [BL]/agonist bioassay)[c]					
			leaves	stems	roots	flowers	bulb	fruit
Epilobium hirsutum	Onagraceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-			
E. montanum		-/-/-	-/-/-	-/-/-	-/-/-	-/-/-		
E. tetragonium		-/-/-	-/0.26/C–	-/0.06/-	-/-/-	-/-/-		
Erigeron alpinus	Compositae (D)	-/-/-	-/0.36/C–		-/0.16/-			
E. glabratus		-/-/-	-/0.33/C–		-/-/-			
E. uniflorus		-/0.11/-	-/0.34/C–	-/0.13/-	-/-/-			
Eryngium maritimum	Umbelliferae (D)	-/0.22/-	14.1/0.35/C–		142/0.91/C+-			
Eucryphia glutinosa	Eucryphiaceae (D)	-/-/-	-/0.23/C–	-/-/-				
Euphorbia platyphyllos	Euphorbiaceae (D)	-/-/-	-/nl/C–	-/-/C–	-/-/-	-/-/C–		
E. villosa		-/-/-	-/-/C–	-/-/-	0.15/-/-			
Frangula (*Rhamnus*) *alnus*	Rhamnaceae (D)	-/-/C–	-/0.07/C–	-/-/-				
Galeopsis speciosa	Labiatae (D)	-/-/-	0.28/0.24/C–	-/-/-	-/-/-			
G. tetrahit		-/-/C–	-/0.19/-	-/-/-	-/-/-	-/-/-		
Galtonia candicans	Hyacinthaceae (M)	-/-/-	-/0.19/C–		-/0.17/-		-/-/-	
G. princeps		-/-/-	-/0.21/C–		-/0.31/-		-/0.14/-	
Garrya elliptica	Garryaceae (D)	-/-/-	-/0.17/-	-/-/-				
G. fremontii		-/-/-	-/-C–	-/-/-				
Geranium maderense	Geraniaceae (D)	-/-/-	-/-/C–	-/-/C–	-/-/C–			
G. molle		-/-/-	-/-/C–	-/-/-		-/-/-		
G. rotundifolium		-/-/-	-/0.44/C–	-/-/C–	-/-/-	-/-/C–		
Geum allepicum	Rosaceae (D)	-/-/-	-/-/C–		-/-/C–			
G. magellanicum		-/-/-	-/-/C–		-/-/C–			
Gilia millefoliata	Polemoniaceae (D)	-/-/C–	-/0.17/C–	-/-/C–	-/-/C–	-/0.14/C–		
Globularia vulgaris	Globulariaceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-			
Grindelia nana	Compositae (D)	-/-/-	-/0.27/C–		-/-/-			
Helleborus foetidissima	Ranunculaceae (D)	12.8/0.39/+-	118/ 3.48/+++-	146/ 1.98/+++-				
Heuchera cylindrica	Saxifragaceae (D)	-/-/-	0.33/0.40/C–		-/-/-			
Hieracium bombycinum	Compositae (D)	-/-/-	-/-/C–		-/-/-			
H. humile		-/-/-	-/-/C–		-/0.22/-			
H. murorum		7.0/0.67/+-	0.27/0.32/C–	-/-/-	-/-/-	-/0.28/C–		
Hydrangea heteromalia	Hydrangaceae (D)	-/-/-	0.24/0.50/C–	-/-/C–	-/-/-			
Hypericum tetrapterum	Guttiferae (D)	-/-/-	-/-/C–	-/-/-	-/-/-	0.51/ 0.39/C–		
Incarvillea forestii	Bignoniacae (D)	-/-/-	-/-/C–		3.6/0.60/C–			
Iris chrysographes	Iridaceae (M)	-/-/-	-/-/-	-/-/-	-/-/-			
I. sibirica		-/-/-	0.32/0.29/C–		-/0.15/-			
*I. spuri*a		-/-/-	0.27/-/C–		0.28/0.26/-			
I. ventricosa		-/-/-	-/-/C–		-/-/-			
Jasminum beesianum	Oleaceae (D)	-/-/-	-/0.34/C–	-/0.16/C–				
Kickxia elatine	Scrophulariaceae (D)	-/-/-	-/0.37/C–	-/-/-	-/-/-	-/-/C–		
Lathyrus sylvestris	Leguminosae-P.[a] (D)	-/-/-	-/-/-	-/-/-	-/-/-			
Lavandula angustifolia	*Labiatae (D)*		-/-/-		0.34/-/C–	0.09/-/-		-/-/-
L. latifolia		-/-/-	-/-/-	-/-/-	-/-/-			
L. multifida		-/-/-	-/-/C–	-/-/-	-/-/-			
L. stoechas		-/-/-	-/0.23/C–	0.13/-/-	-/-/-			
Legousia speculatum-veneris	Campanulaceae (D)	-/-/-	-/0.28/C–	-/-/C–	-/-/-	-/0.25/C–		
Leucanthemum vulgare	Compositae (D)	-/-/-	-/-/C–	-/-/-	-/-/-			
Leucojium nicalense	Amaryllidaceae (M)	-/-/C–	-/-/C–	-/-/C–	-/-/-			
Libertia ixioides	Iridaceae (M)	-/-/-	-/0.50/C–		-/-/-			
Ligustrum congestum	Oleaceae (D)	-/-/-	-/-/C–	-/-/-	14.3/0.25/+-			
Lilium formosanum var. pricei	Liliaceae (M)	-/0.15/-	0.62/0.49/C–		1.05/0.54/-		0.36/ 0.22/-	
L. regale		-/-/-	-/0.61/C–		-/-/-		-/-/-	
Limonium ramosissimum	Plumbaginaceae (D)	3.4/0.60/+-	2.4/0.44/C–		30.7/0.55/++-			
Loasa triphylla	Loasaceae (D)	-/-/C–	-/-/-	-/-/-	-/-/-			-/-/C–
Lobelia syphilitica	Campanulaceae (D)	-/-/-	-/0.29/C–	-/-/C–	0.15/-/-	0.34/ 0.18/C–		
Lycopus americanus	Labiatae (D)	-/-/C–	-/-/C–	-/-/C–	-/-/-	-/0.09/C–		

Table 1. (continued)

Species[a]	Family[b]	Seeds	Ecdysteroid content (RIA [DBL-1]/RIA [BL]/agonist bioassay)[c]					
			leaves	stems	roots	flowers	bulb	fruit
Malus sikkimensis	Rosaceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-			
Malva officinalis	Malvaceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-			
M. robusta		-/-/-	-/0.33/C–	-/-/C–	-/-/-	-/-/C–		
Medicago arabica	Leguminosae-P.[a] (D)	-/-/-	-/-/C–	-/-/C–	-/-/CC–			
Mercurialis annua	Euphorbiaceae (D)	-/-/-	-/0.52/C–	-/-/-	-/-/-	-/-/-		
Mimulus guttatus	Scrophulariaceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-	-/0.20/-		
Myosoton aquaticum	Caryophyllaceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-			
Nepeta camphorata	Labiatae (D)	-/-/-	-/0.13/C–	-/0.09/C–	0.64/0.29/C–	-/0.13/C–		
N. italica		-/-/-	0.31/0.40/C–	0.18/-/-	0.13/-/-	0.24/ 0.11/C–		
N. subsessilis		-/-/-	-/-/C–	-/-/-	-/-/-			
Ochna kirkii	Ochnaceae (D)	-/-/-	0.42/0.31/-	-/-/-	0.17/-/-			
Olearia macrodontata	Compositae (D)	-/-/-	-/0.15/C–	-/-/-				
Orlaya grandiflora	Umbelliferae (D)	-/-/-	-/0.34/C–		-/-/-			
Papaver argemone	Papaveraceae (D)	-/-/-	-/0.23/C–	-/0.14/C–	-/-/-	-/-/C–		
P. dubium		-/-/-	-/-/C–	-/-/-	-/-/-	-/-/C–		
Pelargonium peltatum	Geraniaceae (D)	-/-/-	-/-/C–		-/-/-			
Peltaria alliacea	Cruciferae (D)	0.46/-/-	-/-/C–		-/-/-			
Penstemon azureus	Scrophulariaceae (D)	-/-/C–	0.35/0.34/C–	-/0.17/-	-/0.15/-			
P. laevigatus		-/0.27/C–	0.42/0.10/C–		-/0.26/-			
P. procerus		-/-/-	-/0.13/C–	-/-/-	-/-/-			
P. serrulatus		-/-/-	0.23/0.31/C–	-/0.18/-	-/-/-			
Pittosporum tenuifolium	Pittosporaceae (D)	-/-/-	-/0.23/CC–	2.2/ 0.49/C–	-/-/-			
Plectranthus tomentosus	Labiatae (D)	-/-/-	-/-/C–	-/-/-	-/-/-			
Poa flexuosa	Gramineae (M)	-/-/-	-/-/-		-/-/-	-/-/-		
Potentilla glandulosa	Rosaceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-			
P. pectinata		-/-/-	-/0.20/C–		-/0.53/-			
P. rupestris		-/-/-	-/-/C–		-/-/-			
Primula capitata	Primulaceae (D)	-/-/-	-/0.12/C–		-/-/-			
P. sikkimensis pudibunda		-/-/-	-/-/C–		0.10/-/C–			
P. sino-purpurea		-/-/-	0.49/0.13/C–		-/-/-			
P. wilsonii		-/-/-	-/-/-	-/-/-	-/-/-			
Proboscidea louisianica	Pedaliaceae (D)	-/-/-	-/0.53/C–			-/0.29/C–		-/0.24/C–
Ranunculus arvensis	Ranunculaceae (D)	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-		
R. languinosa		-/-/-	-/-/-		-/-/C–			
Rhododendron albrechtii	Ericaceae (D)	-/-/-	-/-/-	-/-/-	-/-/-			
Rhyneosinapsis (*Coincya*) *monensis*	Cruciferae (D)	-/-/-	-/0.28/C–	-/-/-	-/-/-			
Ribes dikuscha	Grossulariaceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-			
Rivina aurantica	Phytolaccaceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-	-/-/-		
R. humilis		-/-/-	-/-/C–	-/-/-	-/-/-	-/-/-		
Rubus biflorus	Rosaceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-			
Rumex obtusifolius	Polygonaceae (D)	-/-/-	-/0.18/C–	-/-/C–	-/-/-			
Salvia roemeriana	Labiatae (D)	-/-/-	-/0.17/C–	-/-/-	-/-/-	-/-/-		
S. virgata		-/-/-	-/-/-		-/-/-			
Sanguisorba minor	Rosaceae (D)	-/-/-		-/-/C–				
Scabiosa farinosa	Dipsacaceae (D)	-/-/-	-/-/-	-/-/-	-/-/-			
Scrophularia scopolii	Scrophulariaceae (D)	-/-/-	-/0.11/C–	-/-/-	-/-/-			
S. vernalis		-/-/-	0.42/0.37/C–		-/0.12/-			
Sedum album	Crassulaceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-			
S. stoleniferum		-/-/-	0.19/0.17/-	0.13/-/-				
Setaria italica	Gramineae (M)	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-		
Silene pusilla	Caryophyllaceae (D)	4.4/0.75/+-	175/6.58/C+-	183/ 6.50/+-	766/ 49.4/C?			
Sonchus palustris	Compositae (D)	-/-/-	-/-/-	-/-/-	-/-/-			
Sorbus huphensis	Rosaceae (D)	-/-/-	-/-/-	-/-/-	-/-/-			
S. munda		-/-/-	-/-/-	-/-/-	-/-/-			
Spergularia media	Caryophyllaceae (D)	-/-/-	-/0.28/C–	-/-/-	-/-/C–	-/-/-		

Table 1. (continued)

Species [a]	Family [b]	Seeds	Ecdysteroid content (RIA [DBL-1]/RIA [BL]/agonist bioassay) [c]					
			leaves	stems	roots	flowers	bulb	fruit
Stachys annua	Labiatae (D)	0.49/0.26/-	-/0.34/C–	-/0.13/-	-/-/-	-/0.23/C–		
S. byzantina		-/-/-	-/0.33/C–		-/-/-			
Telekia speciosa	Compositae (D)	-/-/-	-/0.14/C–		-/-/-			
Thalictrum flavum	Ranunculaceae (D)	0.15/-/-	-/-/C–	-/-/-	-/0.39/-			
Tragopogon pratensis	Compositae (D)	-/-/-	-/-/C–	-/-/-				
Umbilicus rupestris	Crassulaceae (D)	-/-/-	-/-/C–	-/-/-	-/-/-			
Veronica gentianoides	Scrophulariaceae (D)	-/-/-	-/-/-		-/0.16/-			
Vicia gramineae	Leguminosae-P. [a] (D)	-/-/-	-/-/C–	-/-/C–	-/-/-			
V. orobus		-/-/-	-/-/-	-/-/-	-/-/-			
Wigandia cargcasana	Hydrophyllaceae (D)	-/-/-	-/-/-		-/-/-			

[a] Species and families according to Brummitt [31]. Abbreviations C, M and P for species in the Leguminosae refer to the subfamilies Caesalpinioideae, Mimosoideae and Papilionoideae, respectively.
[b] D, dicotyledonous; M, monocotyledonous.
[c] DBL-1; RIA with the DBL-1 antiserum (µg ecdysone equivalents/g); BL, RIA with the Black antiserum (µg ecdysone equivalents/g); agonist bioassay, activity in agonist version of B_{II} bioassay; -, inactive; +-, active as neat extract; ++-, active as 10-fold dilution; +++, active at 100-fold dilution; C–, cytotoxic as neat extract.

said that the majority of plants which accumulate low levels of ecdysteroids will have the highest levels in the leaves and those which accumulate high levels of ecdysteroids will contain significant levels in all organs.

Since ecdysteroids are detected in species across a wide range of monocotyledonous and dicotyledonous families, the ability to synthesise phytoecdysteroids is apparently a general property of plants. This conclusion is also supported by the observation that 'ecdysteroid-negative' *Zea mays* can convert [2-^{14}C]mevalonic acid and [4-^{14}C]cholesterol into radiolabelled 20-hydroxyecdysone and ecdysone [24].

Occurrence of ecdysteroid conjugates

Ecdysteroid conjugates are common in insect extracts, where their presence is usually qualitatively or quantitatively assessed [25]. A number of ecdysteroid conjugates have been isolated from plant sources [3], but their occurrence has not been systematically assessed. Seed extracts of 290 randomly selected species were assessed for the presence of ecdysteroid conjugates by incubation of the extract with a mixture of hydrolases from the gut juices of *H. pomatia* (table 2). The majority of species remained ecdysteroid-negative after incubation of the seed extracts with the hydrolases, demonstrating that the occurrence of covert conjugates is not a common feature of seed extracts. Further, where significant levels of conjugates were detected, it is in those species (e.g. *H. foetidissima*, *H. murorum*, *S. pusilla*, *Veronica urticifolia*) where significant levels of ecdysteroids were detected prior to hydrolysis. However, from the few studies which have specifically tested for phytoecdysteroid conjugates, it is known that they do not always accompany free ecdysteroids [26]. In a number of extracts with no or low levels of ecdysteroids when incubated without the hydrolases, slightly higher levels of RIA-positive material were detected after incubation with the hydrolases, indicating that low levels of conjugates are present in the extract. However, with other extracts, the RIA response was reduced after incubation with the hydrolases. Neither ecdysone nor 20-hydroxyecdysone is affected by incubation with *Helix* hydrolases [18], but whether other free ecdysteroids could be modified by these enzymes is not known. Furthermore the high proportion of extracts giving a RIA-response may be a consequence of interference in the RIA deriving from the large aliquot size taken for assay.

Can all plants produce ecdysteroids?

Previous initial assessments of seeds and plant material of *A. thaliana* for the presence of phytoecdysteroids had concluded that this species, in common with many other species in the Cruciferae, was ecdysteroid-negative [unpublished data]. We have now assessed the presence of ecdysteroids in individual leaves of over 300 individual plants. While the vast majority of leaf extracts were ecdysteroid-negative, a few extracts proved ecdysteroid-positive. In the case of these extracts (fig. 2), a good linear relationship could be demonstrated between the RIA response and the volume of extract assayed, indicating that the RIA-positive material is ecdysteroidal rather than interfering substances, since only compounds structurally related to the antigen will give a linear response. Thus, while *A. thaliana* is generally ecdysteroid-negative, individual plants within a population do accumulate ecdysteroids (ca 200–800 ng ecdysone eq./g fresh weight). Such levels are not regarded as being effective at deterring insect predators in themselves [4], but may act syner-

Table 2. Effect of incubation with *Helix pomatia* hydrolases on the levels of RIA-positive material in seed extracts. RIA was performed with the DBL-1 antiserum. Species are allocated to Families according to [31]: D = dicot, M = monocot.

Species	Family	µg ecdysone eq./g	
		without hydrolases	with hydrolases
Acaena fissistipula	Rosaceae (D)	–	–
Acer ukurunduense	Aceraceae (D)	–	–
A. barbinerve		–	–
A. mandcluricum		–	–
A. tegmentosum		–	–
Aconitum vulparia	Ranunculaceae (D)	–	–
Actaea asiatica	Ranunculaceae (D)	–	–
A. erythrocarpa		–	–
A. pachypoda		–	–
Agastache foeniculum	Labiatae (D)	–	–
Agrimonia gryposipala	Rosaceae (D)	–	–
A. striata		–	–
Allium albidum	Alliaceae (M)	–	–
A. karataviense		–	–
A. montanum		–	–
Amelanchier florida	Rosaceae (D)	–	–
A. oblongifolia		–	0.378
Anagallis foemina	Primulaceae (D)	–	–
Androsace septemtrionalis	Primulaceae (D)	–	–
Anthemis arvensis	Compositae (D)	–	–
A. punctata		–	–
Aquilegia atrata	Ranunculaceae (D)	–	–
A. canadensis		–	–
A. flavescens		–	–
A. nevadensis		–	–
Arabis drummondii	Cruciferae (D)	–	–
A. soyrei		–	–
Arenaria obtusiloba	Caryophyllaceae (D)	–	–
Argemone mexicana	Papaveraceae (D)	–	–
Arisaema erubescens	Araceae (M)	–	–
A. franchianum		–	–
Arnica mollis	Compositae (D)	–	0.248
Arum maculatum	Araceae (M)	–	–
Aruncus diocus	Rosaceae (D)	–	0.155
Asarina barclaiana	Scrophulariaceae (D)	–	–
A. erubescens		0.176	0.456
A. procumbens		–	–
A. purpusii		–	–
Asclepias speciosa	Asclepiadaceae (D)	–	–
Asparagus schoberioides	Asparagaceae (M)	–	–
Aster conspicuus	Compositae (D)	–	–
A. nataalensis		–	–
A. pyrenaeus		–	–
Astilbe grandis	Saxifragaceae (D)	–	–
A. koreana		–	–
A. myriantha		–	–
A. thunbergia var. *congesta*		–	–
Bellevalia romana	Hyacinthaceae (M)	–	–
Bensoniellia oregona	Saxifragaceae (D)	–	–
Berberis integerima	Berbericaceae (D)	–	–
Bergenia stracheyi	Saxifragaceae (D)	1.634	0.086
Betula lenta	Betulaceae (D)	–	–
Buglossoides arvensis	Boraginaceae (D)	–	–
Cacalia suaveolens	Compositae (D)	–	–
Callistemon pityoides	Myrtaceae (D)	–	–
Campanula saxifraga	Campanulaceae (D)	–	–

Table 2. (continued)

Species	Family	µg ecdysone eq./g	
		without hydrolases	with hydrolases
Arabis (Cardaminopsis) arenosa	Cruciferae (D)	–	–
Cardiocrinum cordata	Liliaceae (M)	–	–
C. giganteum		–	–
C. yunnanensis		–	–
Carex berggrenis	Cyperaceae (M)	–	–
C. flagellifera		–	–
Celmisia webbii	Compositae (D)	–	–
Chaenomeles cathayensis		–	–
Chaenorhinum minus	Scrophulariaceae (D)	–	–
Chelone lyonii	Scrophulariaceae (D)	–	–
Chrysanthemum coronarium	Compositae (D)	–	–
Cimifuga europaea	Ranunculaceae (D)	–	–
Cistus albidus	Cistaceae (D)	–	–
C. hirsutus		–	–
C. populifolius		–	–
Clematis chrysocoma	Ranunculaceae (D)	–	–
C. maximowicziana var. *robusta*		–	–
C. peterae		–	–
Cleome serrulata	Capparaceae (D)	–	–
Clethra arborea	Clethraceae (D)	1.838	1.740
Consolida ambigua	Ranunculaceae (D)	–	–
Coreopsis major	Compositae (D)	–	–
Coriaria microphylla	Coriariaceae (D)	–	–
Corydalis bulbosa	Papaveraceae (D)	–	–
C ophiocarpa		–	–
Cotoneaster melanocarpus var. *laxiflorus*	Rosaceae (D)	–	–
C. racemiflorus var. *songaricus*		–	–
C. zabelii		–	–
C. zeravschanicus		–	–
Cynoglossum hungaricum	Boraginaceae (D)	–	–
Daphne mezereum	Thymelaeaceae (D)	–	–
Delphinium cuneatum	Ranunculaceae (D)	–	0.202
Desmodium elegans	Leguminosae-P. (D)	–	–
Dianthus amurensis	Caryophyllaceae (D)	–	–
D. hungaricus		–	–
D. shinanensis		–	–
Digitalis grandiflora	Scrophulariaceae (D)	–	–
Dipsacus japonicus	Dipsacaceae (D)	–	0.272
Draba languinosa	Cruciferae (D)	–	–
Dracocephalum nutans	Labiatae (D)	0.320	0.124
D. purdomii		0.275	0.241
Dryas drummondii	Rosaceae (D)	0.182	0.257
Epilobium hirsutum	Onagraceae (D)	–	–
E. montanum		–	–
E. paniniculatum		–	–
E. tetragonium		–	–
Erigeron alpinus	Compositae (D)	–	–
E. glabratus		–	–
E. multiradiatus		0.065	0.441
E. uniflorus		0.307	0.351
Eryngium maritimum	Umbelliferae (D)	0.162	0.211
Euonymus atropurpureus	Celastraceae (D)	0.443	0.300
Euphorbia myrsinites	Euphorbiaceae (D)	0.370	0.277
E. platyphyllos		0.369	0.720

Table 2. (continued)

Species	Family	µg ecdysone eq./g without hydrolases	µg ecdysone eq./g with hydrolases
E. villosa		0.486	0.342
Filipendula ulmaria	Rosaceae (D)	0.053	0.282
F. vulgaris		0.275	0.229
Frangula (*Rhamnus*) *alnus*	Rhamnaceae (D)	–	–
Fritillaria acmopetala	Liliaceae (M)	–	–
F. involucrata		–	–
Galeopsis speciosa	Labiatae (D)	0.363	0.257
Galium sylvaticum	Rubiaceae (D)	0.215	0.233
Galtonia candicans	Hyacinthaceae (M)	0.105	0.192
G. princeps		0.289	0.434
Garrya fremontii	Garryaceae (D)	0.231	–
Gentiana kuroo	Gentianaceae (D)	0.659	0.251
G. saponaria		0.352	0.192
G. saxosa		0.313	0.282
Geranium molle	Geraniaceae (D)	0.512	0.622
G. rotundifolium		0.297	0.445
Geum allepicum	Rosaceae (D)	0.472	0.506
G. canadense		0.597	0.388
G magellanicum		0.214	0.199
G. montanum		0.339	0.474
Globularia punctata	Globulariaceae (D)	0.579	0.372
G. vulgaris		0.366	0.293
Grindelia nana	Compositae (D)	2.58	–
Gunnera tinctoria	Gunneraceae (D)	0.368	0.368
Helleborus foetidissima	Ranunculaceae (D)	6.36	14.51
Heuchera cylindrica	Saxifragaceae (D)	–	–
H. pilosa		–	–
Hieracium bombycinum	Compositae (D)	0.267	–
H. humile		0.679	0.841
H. laevigatum		0.299	0.399
H. murorum		9.48	12.64
Hladnikia pastinacifolia	Umbelliferae (D)	0.521	0.196
Hyacinthoides italica	Hyacinthaceae (M)	0.347	0.315
H. non–scripta		0.213	0.334
Hydrangea heteromalia	Hydrangaceae (D)	–	–
Hyomecon vernalis	Papaveraceae (D)	0.586	0.196
Hypericum maculatum	Guttiferae (D)	0.391	0.358
H. tetrapterum		0.439	–
Icarvillea forestii	Bignoniaceae (D)	0.641	0.405
Iris chrysographes	Iridaceae (M)	0.344	–
I. sibirica		0.416	0.451
I. spuria		–	–
I. ventricosa		0.294	–
Jasione jankae	Campanulaceae (D)	–	–
Jaminum beesianum	Oleaceae (D)	0.651	0.274
Jeffersonia diphylla	Berberidaceae (D)	0.388	–
Kickxia elatine	Scrophulariaceae (D)	0.304	–
Lathyrus niger	Leguminosae–P. (D)	0.292	–
Lavandula angustifolia	Labiatae (D)	0.352	–
L. stoechas		–	–
Legousia speculatum-veneris	Campanulaceae (D)	0.293	0.293 0.293
Leucantheum maximum	Compositae (D)	–	–
Leucojium nicalense	Amaryllidaceae (M)	0.283	–
Libertia ixioides	Iridaceae (M)	–	3.20
Lilium carnolicum	Liliaceae (M)	0.196	–

Table 2. (continued)

Species	Family	µg ecdysone eq./g without hydrolases	µg ecdysone eq./g with hydrolases
L. formosanum var. *pricei*		0.357	0.260
L. pilosiusculum		0.476	0.037
L. regale		–	–
Lobelia syphilitica	Campanulaceae (D)	0.401	–
Lonicera henryi	Caprifoliaceae (D)	1.86	0.504
L. maximowiczii		0.631	0.232
L. pileata		0.268	0.179
Lycopus americanus	Labiatae (D)	1.30	0.357
Mahonia nervosa	Berberidaceae (D)	0.305	0.458
Malus sargintii	Rosaceae (D)	0.217	0.278
M. toringoides		–	–
Malva officionalis	Malvaceae (D)	–	–
M. robusta		–	–
Medicago arabica	Leguminosae–P. (D)	–	–
Mentzelia nuda	Loasaceae (D)	–	–
Mercurialis annua	Euphorbiaceae (D)	–	–
Mimulus guttatus	Scrophulariaceae (D)	–	–
Mitella pentandra	Saxifragaceae (D)	–	–
Muscari conicum	Hyacinthaceae (M)	–	–
M. neglectum		–	–
Musschia aurea	Campanulaceae (D)	–	–
Myosoton aquaticum	Caryophyllaceae (D)	–	–
Nectaroscordum siculum	Alliaceae (M)	–	–
Nepeta camphorata	Labiatae (D)	–	–
N. italica		0.426	–
N. nawachinii		–	–
Ochna kirkii	Ochnaceae (D)	–	–
Olearia macrodontata	Compositae (D)	–	–
Orlaya grandiflora	Umbelliferae (D)	0.446	0.507
Ornithogalum caudatum	Hyacintaceae (M)	–	–
Padus (*Prunus*) *maackii*	Rosaceae (D)	–	–
Paeonia daurica	Paeoniaceae (D)	–	–
P. obovata		–	–
Palisota bracteosa	Commelinaceae (M)	–	–
Papaver argemone	Papaveraceae (D)	0.534	0.356
P. dubium		–	–
Pelargonium peltatum	Geraniaceae (D)	–	–
Peltaria alliacea	Cruciferae (D)	0.952	0.811
Penstemon azureus	Scrophulariaceae (D)	–	–
P. laevigatus		0.749	0.652
P. lyallii		–	–
P. newberryi		–	–
P. procerus		–	–
P. serrulatus		–	–
Petalostemon (*Dalea*) *purpureum*	Leguminosae-P. (D)	0.647	0.370
Peucedanum morisonii	Umbelliferae (D)	–	–
P. palustre		–	–
Phlomis russelliana	Labiatae (D)	–	–
P. tuberosa		–	0.098
Phyteuma orbiculare	Campanulaceae (D)	–	–
Pieris formosa	Ericaceae (D)	–	–
Pittosporum tenuifolium	Pittosporaceae (D)	–	–
Polygonatum iodoratum var. *maximowiczii*	Convallariaceae (M)	–	1.38
P. latifolium		–	–
Potentilla glandulosa	Rosaceae (D)	–	–
P. pectinata		–	–

Table 2. (continued)

Species	Family	µg ecdysone eq./g	
		without hydro-lases	with hydro-lases
P. rupestris		–	–
Primula capitata	Primulaceae (D)	–	–
P. sikkimensis pudibunda		–	1.36
P. sino-purpurea		–	0.181
Proboscidea louisianica	Pedaliaceae (D)	0.659	0.078
Pyrus ussuriensis	Rosaceae (D)	0.311	–
Ranunculus languinosa	Ranunculaceae (D)	0.120	0.211
Rhamnus davurica	Rhamnaceae (D)	–	–
Rhododendron oroetrephes	Ericaceae (D)	–	–
Rhus verniciflua	Anacardiaceae (D)	0.137	–
Ribes alpinum	Grossulariaceae (D)	–	–
R. dikuscha		–	–
Rivina aurantiaca	Phytolaccaceae (D)	–	–
Rosa blanda	Rosaceae (D)	–	–
R. glauca		–	–
R. pendula		–	–
R. villosa		–	–
Salvia verticillata	Labiatae (D)	0.363	0.435
S. arizonia		0.324	0.036
S. glutinosa		–	–
S. pratensis f. *rubicunda*		–	–
Sanguisorba minor	Rosaceae (D)	–	–
S. sitchensis		–	–
Saxifraga grisebachii	Saxifragaceae (D)	–	–
S. paniculata		–	–
S. rotundifolia		–	–
Scabiosa canescens	Dipsacaceae (D)	–	–
Schizanthus hookerianus	Solanaceae (D)	–	–
Scrophularia scopolii	Scrophulariaceae (D)	–	–
S. vernalis		–	–
Sedum album	Crassulaceae (D)	–	–
S. pilosum		–	–
S. stoleniferum		–	–
Serratula sogdiana	Compositae (D)	23.3	17.2
Setaria italica	Gramineae (M)	0.606	0.720
Sherardia arvensis	Rubiaceae (D)	–	–
Silene pulsilla	Caryophyllaceae (D)	9.42	12.40
Sisyrinchium montanum	Iridaceae (M)	–	–
S. patagonicum		–	–
Smilacina (*Maianthemum*) *racemosa*	Convallariaceae (M)	–	–
Sonchus palustris	Compositae (D)	–	–
Sorbaria sorbifolia	Rosaceae (D)	0.547	0.547
Spergularia media	Caryophyllaceae (D)	–	–
Stachys alopecuros	Labiatae (D)	0.342	0.962
S. annua		0.217	0.495
S. byzantina		0.228	0.471
S. recta		–	–
S. sylvanica		0.057	0.497
Telekia speciosissima	Compositae (D)	–	–
Telesonix (*Boykinia*) *jamesii*	Saxifragaceae (D)	–	–
Teucrium arduinii	Labiatae (D)	–	–
T. scorodonia		–	0.853
Thalictrum flavum	Ranunculaceae (D)	–	–
T. revolutum		–	–
Tragopogon pratensis	Compositae (D)	–	–

Table 2. (continued)

Species	Family	µg ecdysone eq./g	
		without hydro-lases	with hydro-lases
Triosteum erythrocarpum	Caprifoliaceae (D)	–	–
Trollius chinensis	Ranunculaceae (D)	0.294	0.518
Veratrum lobelianum	Melianthiaceae (M)	–	–
Verbascum nevadense	Scrophulariaceae (D)	–	–
Veronica urticifolia	Scrophulariaceae (D)	4.69	6.43
Vincetoxicum hirundinaria	Asclepiadaceae (D)	0.743	0.314
Viola mirabilis	Violaceae (D)	–	–

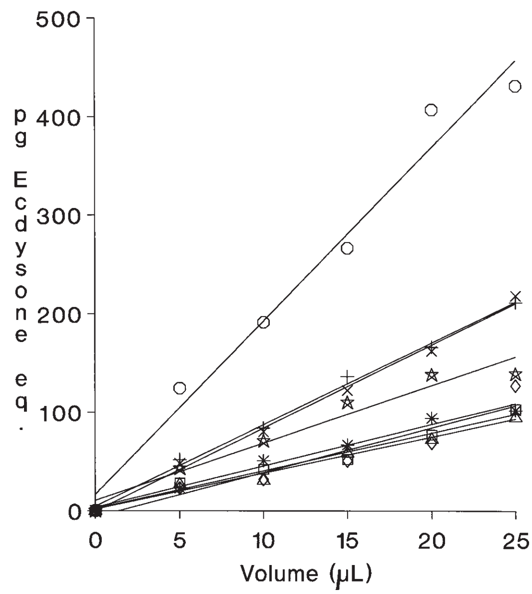

Figure 2. Relationship between RIA response and aliquot size for extracts of individual leaves from plants of *Arabidopsis thaliana*. Individual freeze-dried leaves were extracted in 1 ml methanol and the extracts assessed by RIA: (☆) plant 5/2, (+) plant 5/3, (*) plant 5/4, (□) plant 6/1, (×) plant 6/10, (◇) plant 8/1, (△) plant 8/2 and (○) plant 8/4.

gistically with other defence chemicals to provide protection.

To test further 'ecdysteroid-negative' species, extracts of 50 species were partially purified and concentrated. RIA was used to determine whether these samples contained detectable levels of ecdysteroids. Almost all the samples (48/50) were RIA-positive (table 3) and, further, the response was linearly related to sample size (fig. 3), indicating strongly that the response was a consequence of the presence of ecdysteroids. The other two samples generated non-linear positive responses, which may indicate the presence of interfering substances in addition to ecdysteroids. The levels present in the majority of these concentrated samples were generally in the region of 20–150 ng ecdysone equivalents/g dry weight. Why the extract of *Anthemis arvensis* (1436 ng ecdysone eq./g) was not revealed as ecdysteroid-positive in the initial screening and *Helix* hydrolysis experiment (table 2) is not clear, as the level found after concentration is above the original detection limit, but presumably partial purification has removed substances which interfere in the RIA.

Implications

The finding that individual plants of *A. thaliana* (Cruciferae) may accumulate low levels of ecdysteroids indicates that even species within genera, tribes or families which are not known to contain ecdysteroid-containing species retain the capacity to synthesise and accumulate ecdysteroids (albeit at a low level). Thus, one must conclude that most (if not all) plants retain this capacity, even if in 95% of species, this pathway is very poorly activated. The data presented here raise the question whether this individual variation in *A. thaliana* is dependent on genetic or environmental factors. However, the well-characterised genome of this species [27, 28] offers distinct advantages to elucidate this question and to study phytoecdysteroid biosynthesis and its regulation.

The universality of phytoecdysteroid presence in plants is supported by the detection of low levels of ecdysteroid immunoassay-positive material in concentrated extracts

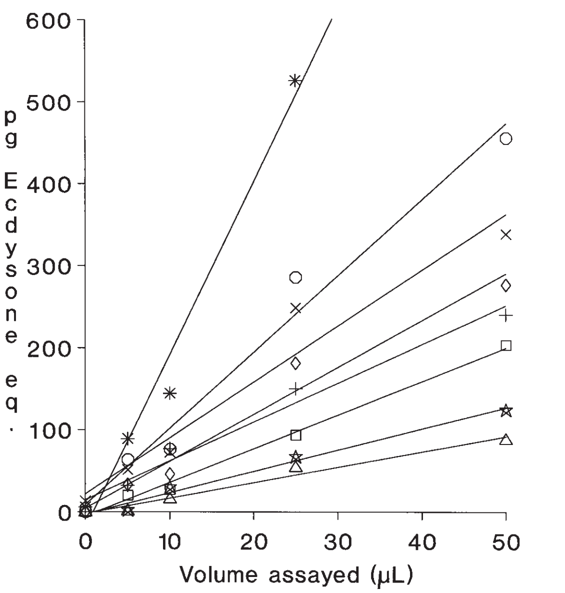

Figure 3. Relationship between RIA response and aliquot size for concentrated extracts of seeds of representative “ecdysteroid-negative” species. Seed extracts (2.5 mL; equivalent to ca. 14.5 mg seeds) were partially purified by reversed-phase solid-phase extraction cartridges and the 75% methanol fraction concentrated to 250 µL. Aliquots of this concentrate were assessed by RIA (DBL-1 antiserum): (✻) *Allium albidum*, (○) *Aconitum vulparia*, (×) *Argemone mexicana*, (◇) *Bellevalia romana*, (+) *Agastache foeniculum*, (□) *Alemanchier florida*, (☆) *Chaenorhinum minus* and (△) *Arabis arenosa*.

Table 3. Assessment of the levels of RIA-positive material in “ecdysteroid-negative” seed extracts after 100-fold concentration of the extracts.

Species	Family	ng ecdysone eq./g
Acaena fissistipula	Rosaceae (D)	311
Acer mandcluricum	Aceraceae (D)	68
Aconitum vulparia	Ranunculaceae (D)	162
Actaea erythrocarpa	Ranunculaceae (D)	68
Agastache foeniculum	Labiatae (D)	82
Agrimonia gryposipala	Rosaceae (D)	112
Allium albidum	Alliaceae (M)	317
Amelanchier florida	Rosaceae (D)	60
Anagallis foemina	Primulaceae (D)	123
Anthemis arvensis	Compositae (D)	1436
Aquilegia atrata	Ranunculaceae (D)	6
Arabis drummondii	Cruciferae (D)	46
Arenaria obtusiloba	Caryophyllaceae (D)	36
Argemone mexicana	Papaveraceae (D)	124
Arisaema erubescens	Araceae (M)	51
Asarina barclaiana	Scrophulariaceae (D)	103
Asclepias speciosa	Asclepiadaceae (D)	126
Asparagus schoberioides	Asparagaceae (M)	74
Aster conspicuus	Compositae (D)	84
Astilbe grandis	Saxifragaceae (D)	33
Bellevalia romana	Hyacinthaceae (M)	113
Bensoniellia oregona	Saxifragaceae (D)	38
Berberis integerima	Berbericaceae (D)	30
Betula lenta	Betulaceae (D)	72
Buglossoides arvensis	Boraginaceae (D)	117
Cacalia suaveolens	Compositae (D)	101
Callistemon pityoides	Myrtaceae (D)	14
Campanula saxifraga	Campanulaceae (D)	117
Arabis (Cardaminopsis) arenosa	Cruciferae (D)	23
Cardiocrinum cordata	Liliaceae (M)	29
Carex berggrenis	Cyperaceae (M)	NL
Celmisia webbii	Compositae (D)	72
Chaenomeles cathayensis	Rosaceae (D)	NL
Chaenorhinum minus	Scrophulariaceae (D)	35
Chrysanthemum coronarium	Compositae (D)	202
Cimifuga europaea	Ranunculaceae (D)	44
Cistus albidus	Cistaceae (D)	25
Clematis chrysocoma	Ranunculaceae (D)	15
Cleome serrulata	Capparaceae (D)	49
Consolida ambigua	Ranunculaceae (D)	29
Coreopsis major	Compositae (D)	37
Coriaria microphylla	Coriariaceae (D)	88
Corydalis bulbosa	Papaveraceae (D)	49
Cotoneaster melanocarpus var. *laxiflorus*	Rosaceae (D)	30
Cynoglossum hungaricum	Boraginaceae (D)	36
Daphne mezereum	Thymelaeaceae (D)	32
Desmodium elegans	Leguminosae-P. (D)	77
Dianthus amurensis	Caryophyllaceae (D)	77
Digitalis grandiflora	Scrophulariaceae (D)	40
Dipsacus japonicus	Dipsacaceae (D)	87

RIA was performed with the DBL-1 antiserum. Species are allocated to families according to [31]: D = dicot, M = monocot, NL = non-linear response.

of 'ecdysteroid-negative' species. Ecdysteroids have considerable potential for the protection of crop species against invertebrate predators, or, alternatively, plants might be efficient sources of these compounds for pharmaceutical/medical applications [6]. Thus, elevation of phytoecdysteroid levels in ecdysteroid-poor species will not require the introduction of genes corresponding to the enzymes of the biosynthetic pathway. Rather, levels can be altered by altering the regulation of expression of existing genes. This is a much more feasible goal, which could be achieved by traditional breeding strategies or genetic modification.

Ecdysteroid levels in *S. oleracea* are inducible by mechanical or insect damage of roots [29, 30]. This appears to be mediated by jasmonate signalling pathways [29]. Thus, the application of appropriate elicitors may be a further way to elevate ecdysteroid levels in crop species.

The same situation probably applies to other classes of secondary products, including those with direct medicinal applications, such that elucidation of the regulation of the biosynthetic pathways may enable strategies to elevate levels in convenient crop species and thus facilitate the use of plants as 'cell-factories' for the many pharmaceuticals of plant origin.

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1 Koolman J. (ed.) (1989) Ecdysone: from Chemistry to Mode of Action, Thieme, Stuttgart
2 Bergamasco R.and Horn D. H. S. (1983) Distribution and role of insect hormones in plants. In: Endocrinology of Insects, pp. 627–654, Downer R. G. H. and Laufer H. (eds), Liss, New York
3 Lafont R.and Wilson I.D. (1996) The Ecdysone Handbook, 2nd edn., The Chromatographic Society, Nottingham
4 Dinan L. (1998) A strategy towards the elucidation of the contribution made by phytoecdysteroids to the deterrence of invertebrate predators on plants. Russ. J. Plant Physiol. **45:** 296–305
5 Sláma K. and Lafont R. (1995) Insect hormones – ecdysteroids: their presence and actions in vertebrates. Eur. J. Entomol. **92:** 355–377
6 Dinan L., 2001. Phytoecdysteroids: biological aspects. Phytochemistry **57:** 325–339
7 Báthory M., Tóth I., Szendrei K. and Reisch J. (1982) Ecdysteroids in *Spinacia oleracea* and *Chenopodium bonus-henricus*. Phytochemistry **21:** 236–238
8 Grebenok R.J., Ripa P. V. and Adler J.H. (1991) Occurrence and levels of ecdysteroids in spinach. Lipids **26:** 666–668
9 Dinan L. (1992) The association of phytoecdysteroids with flowering in fat hen, *Chenopodium album*, and other members of the Chenopodiaceae. Experientia **48:** 305–308
10 Dinan L. (1995) Distribution and levels of phytoecdysteroids within individual plants of the species of the Chenopodiaceae. Eur. J. Entomol. **92:** 295–300
11 Lafont R. (1997) Ecdysteroids and related molecules in animals and plants. Arch. Insect Biochem. Physiol. **35:** 3–20
12 Imai S., Toyosato T., Sakai M., Sato Y., Fujioka S., Murata E. et al. (1969) Screening results of plants for phytoecdysones. Chem. Pharm. Bull. **17:** 335–339
13 Dinan L. (1995) A strategy for the identification of ecdysteroid receptor agonist and antagonists from plants. Eur. J. Entomol. **92:** 271–283
14 Dinan L., Whiting P. and Scott A. (1998) Taxonomic distribution of phytoecdysteroids in seeds of members of the Chenopodiaceae. Biochem. Syst. Ecol. **26:** 553–576
15 Zibareva L. (2000) Distribution and levels of phytoecdysteroids in plants of the genus *Silene* during development. Arch. Insect Biochem. Physiol. **43:** 1–8
16 Lafont R., Bouthier A. and Wilson I.D. (1991) Phytoecdysteroids: structures, occurrence, biosynthesis and possible ecological significance. In: Insect Chemical Ecology, pp. 197–214, Hrdy I. (ed.), Academia, Prague
17 Dinan L., Hormann R.E. and Fujimoto T. (1999) An extensive ecdysteroid CoMFA. J. Comput. Aided Mol. Design **13:** 185–207
18 Dinan L., Bourne P. and Whiting P, (2001) Phytoecdysteroid profiles in seeds of *Sida* spp. (Malvaceae). Phytochem. Anal. **12:** 110–119
19 Clément C.Y., Bradbrook D.A., Lafont R. and Dinan L. (1993) Assessment of a microplate-based bioassay for the detection of ecdysteroid-like or antiecdysteroid activities. Insect Biochem. Mol. Biol. **23:** 187–193
20 Dinan L. (1992) The analysis of phytoecdysteroid in single (pre-flowering stage) specimens of fat hen, *Chenopodium album*. Phytochem. Anal. **3:** 132–138
21 Whiting P. and Dinan L. (1988) The occurrence of apolar ecdysteroid conjugates in newly-laid eggs of the house cricket, *Acheta domesticus*. J. Insect Physiol. **34:** 625–631
22 Harmatha J. and Dinan L. (1997) The biological activity of natural and synthetic ecdysteroids in the B_{II} bioassay. Arch. Insect Biochem. Physiol. **35:** 219–225
23 Christen P. (1989) Withanolide: Naturstoffe mit vielversprechendem Wirkungsspektrum. Pharm. Unserer Zeit **18:** 129–139
24 Devarenne T.P., Sen-Michael B. and Adler J.H. (1995) Biosynthesis of ecdysteroids in *Zea mays*. Phytochemistry **40:** 1125–1131
25 Rees H.H and Isaac R.E. (1985) Biosynthesis and metabolism of ecdysteroids and methods of isolation and identification of the free and conjugated compounds. Methods Enzymol. **111B:** 377–410
26 Meng Y., Whiting P., Zibareva L., Bertho G., Girault J.-P., Lafont R. et al. (in press) Identification and quantitative analysis of the phytoecdysteroids in *Silene* species (Caryophyllaceae) by high-performance liquid chromatography: novel ecdysteroids from *S. pseudotites*. J. Chromatogr.
27 Kaul S., Koo H.L., Jenkins J., Rizzo M., Rooney T., Tallon L.J. et al. (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408:** 796–815
28 Tabata S., Kaneko T., Nakamura Y., Kotani H., Kato T., Asamizu E. et al. (2000) Sequence and analyis of chromosome 5 of the plant *Arabidopsis thaliana*. Nature **408:** 823–826
29 Schmelz E.A., Grebenok R.J., Galbraith D.W. and Bowers W.S. (1998) Damage-induced accumulation of phytoecdysteroids in spinach: a rapid root response involving the octadecanoic acid pathway. J. Chem. Ecol. **24:** 339–360
30 Schmelz E.A., Grebenok R.J., Galbraith D. W. and Bowers W.S. (1999) Insect-induced synthesis of phytoecdysteroids in spinach, *Spinacia oleracea*. J. Chem. Ecol. **25:** 1739–1757
31 Brummitt R.K. (1992) Vascular Plant Families and Genera, Royal Botanic Gardens, Kew