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Developing Inflorescences of Male and Female *Rumex acetosa* L. Show Differences in Gibberellin Content

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Abstract

Rumex acetosa L. (common sorrel) is a dioecious perennial in the family Polygonaceae. Gibberellins (GAs) of the early 13-hydroxylation pathway and the putative early 3β, 13-hydroxylation pathway were previously identified in young *R. acetosa* inflorescences by GC-MS. In this investigation to examine the GA content of individual inflorescences ELISAs were used for quantitative analysis. Significant differences were revealed between the sexes in the GA content of young inflorescences, and GC-SRM was used to validate the observed trends. Males had higher levels of the 3β, 13-hydroxylated C_{20}-GA GA_{18} and the 2β, 13-hydroxylated C_{19}-GA GA_{29}, whereas females had higher levels of the 13-hydroxylated C_{20}-GAs GA_{53} and GA_{19}. It is suggested that the conversion from C_{20}-GAs to C_{19}-GAs is under tighter control in the inflorescences of females compared to male plants and therefore there is accumulation of the C_{20}-GAs in the females. Results from flowering bioassays using authentic GAs indicate that differences in GA content between the sexes are unlikely to be a consequence of sex determination.

Key words: *Rumex acetosa*; Polygonaceae; GC-SRM; ELISA; Inflorescences; Gibberellins; Early 13-hydroxylation pathway; Early 3β, 13-hydroxylation pathway; Sex determination

Introduction

Most angiosperms are hermaphrodites and flowers containing both male and female sexual organs develop on all plants. Some plants have evolved dioecy, that is, separate male (staminate) and female (pistillate) flowers on different individuals, absolutely preventing self-pollination. In addition, dioecy helps to promote sexual specialization and allows resources to be utilized more efficiently. For example, in hermaphrodites, the production of pollen may limit the production of ovules and *vice versa*, a limitation that is avoided in dioecious plants (Dellaporta and Calderon-Urrea 1993; Ainsworth 2000).

Rumex acetosa L. (common sorrel) is one of only two wholly dioecious species in the family Polygonaceae native to the UK (Putwain and Harper 1972) and is found in diverse habitats. It has a rosette growth habit, and in the vegetative phase, hastate leaves with characteristic extended basal

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lobes and long petioles (Clapham and others 1981; Lousley and Kent 1981).

In late spring both males and females initiate racemose inflorescences, which are composed of flowers borne in clusters at nodes along the upper part of the inflorescence stem. The outer two floral whorls of both male and female flowers contain perianth organs and consist of three small sepaloid segments each, with no obvious differences between sepals and petals. The inner floral whorl contains the reproductive organs. In the male flower six stamens are found in the inner whorl and in the female flower the gynoecium, consisting of three fused carpels, occupies the inner whorl (Ainsworth and others 1998).

It is when flowers are open, revealing the stamens and styles in male and female flowers, respectively, that the primary phenological differences between the plants are most obvious to the unaided sans microscope eye. Using scanning electron microscopy (SEM) Ainsworth and others (1995) studied the morphological changes during floral development in male and female flowers. They observed that stamen primordia were initiated in the female flower, but arrested in development at an early stage and obscured from view by the developing gynoecium.

Sex determination is the regulation of sexual differentiation (Durand and Durand 1991) and researchers have long been interested in the mechanisms by which sex is determined in dioecious plants. In a few dioecious species such as *R. acetosa* there is a chromosomal basis to dioecy through the occurrence of sex chromosomes, with females having 12 autosomes and two X chromosomes (2n = 12 + XX) and males having 12 autosomes, one X chromosome and two Y chromosomes (2n = 12 + XY_1Y_2). Sex determination in *R. acetosa* is influenced by the X:autosome set ratio. Female plants have a ratio of 1.0 or higher, whereas males have a ratio of 0.5 or lower. In hermaphrodite plants, which occur infrequently, a ratio of 0.5 to 1.0 has been found (Parker 1990; Parker and Clark 1991).

Following the discovery of the critical roles played by hormones such as androgens and estrogens in sex differentiation in animals, researchers examined the influence of such animal hormones (Löve and Löve 1945) and subsequently plant growth regulators such as cytokinins, GAs, ethylene and auxins on sex determination in plants. The approaches commonly adopted include observation of the effects of exogenous applications of plant growth regulators on the sex expression of flowers (Negi and Olmo 1966; Chailakhyan and Khryanin 1979; Champault and others 1985; Durand and Durand 1991) and quantification of the endogenous content of specific plant growth regulators (Dauphin-Guerin and others 1980; Hautala and others 1986; Jelič and others 1988; Louis and others 1990; De Kock and others 1994).

The effects of GAs on the sex determination of unisexual plants have primarily been investigated with regard to monoecious plants, in such plants, staminate and pistillate flowers are found on the same individuals. Exogenous application of GAs promoted the formation of male flowers in several members of the Cucurbitaceae such as *Cucumis sativa* and *Luffa acutangula* (Fuchs and others 1977; Chailakhyan and Khrianin 1987). Although for most monoecious plants GAs are primarily associated with masculinization, with some species such as *Zea mays* application of GAs promoted feminization (Krishnamoorthy and Talukdar 1976). The normal sex determination process in maize requires stamen development to be supressed in ear florets and carpel development to be supressed in tassel florets (Irish 1999) and in wild-type plants, GA levels are higher in the ears than the tassels (Rood and others 1980).

The involvement of GAs in the sex determination of dioecious plants has only been investigated in a few species. Chailakhyan and Khrianin (1987) examined the effects of exogenous GA_3 on the sex expression of two dioecious species: *Spinacia oleracea* and *Cannabis sativa*. In both species GA_3 promoted male sex expression. In the breeding of kiwi fruit (*Actinidia deliciosa*), because of the yield loss due to male individuals, a primary objective is the selection of hermaphrodite clones. Marchetti and others (1992) found GAs to be the most effective plant growth regulators for altering floral sex expression in this species. In females treatment with GA_3 increased the number of styles in the flowers and in males GA treatment induced the development of styles.

Early studies used bioassays to measure the endogenous GA activity in male and female individuals in dioecious species. Lesham and Ophir (1977) analyzed the endogenous GA content of the inflorescences and foliage from two dioecious tree species—*Ceratonia siliqua* and *Phoenix dactylifera*. The barley endosperm assay was used to analyze GA activity and in both species females had higher levels of GA activity than males. Also, using barley endosperm bioassays, Čulafič and Neškovič (1976) found quantitative differences in GA activity between males and females of *Rumex acetosella*. During the rosette stage, a higher level of activity was found in extracts of female shoots, whereas analysis of plants just prior to the opening of florets in the inflorescence revealed that males had higher levels of

GA activity. Čulafič and Neškovič (1974) also looked at GA levels in male and female *Spinacia oleracea* plants when the first flowers were visible. Using thin layer chromatography to separate the GAs and the barley endosperm bioassay to assay activity, they found that although the total content of GAs was similar for the two sexes, females had higher levels of a compound that had a similar retention factor (Rf) to that of GA_3, whereas males had higher levels of compounds with Rf values corresponding to GA_5 and GA_{4+7}. One of the problems with the use of bioassays is that GAs do not have the same activity in a particular bioassay as they would in the species in which they were identified. Also, in addition to being a relatively slow process, bioassays by definition can only detect biologically active compounds (Wang and others 1985) and inactive GAs are not accounted for.

Using gas chromatography-mass spectrometry (GC-MS) Rijnders and others (1997) found GAs of both the early 13-hydroxylation pathway and the non-13-hydroxylation pathway in shoots of *R. acetosa*. Analyzing tissue samples from male and female plants separately with full scan GC-MS, Stokes and others (2003) found GAs of the early 13-hydroxylation pathway and the early 3β, 13-hydroxylation pathway in *R. acetosa* inflorescences (Figure 1). Some qualitative differences were observed between the sexes in their complement of GAs. Although, like the related species *Rumex acetosella*, *R. acetosa* is not a sexually labile species (Bavrina and others 1991; Čulafič 1999; Ainsworth 2000) the hypothesis in the present study was that the inflorescence tissues from plants of different sexes would also show quantitative differences in their endogenous GA content. Therefore, to gain a better appreciation of hormone metabolism in this dioecious species, enzyme-linked immunosorbent assays (ELISAs) and gas chromatography-selected reaction monitoring (GC-SRM) were used to compare the GA content of young inflorescences from male and female plants. As a first step to understanding whether such observed differences in GA content were a cause or a result of the sex determination process, flowering bioassays were carried out using authentic GAs of the little-studied early 3β, 13-hydroxylation pathway.

Materials and Methods

Quantitative Analysis Using ELISAs

Plant Material. The plants used in this study were F1 generation progeny of crosses carried out on native *R. acetosa* plants collected from various sites in Cornwall, UK, by Dr. Anthony Lowe, University of Cambridge. The F1 generation seed from these crosses of native *R. acetosa* plants was collected in August 1997 and immediately germinated in plug-trays in an unheated experimental glasshouse. Plants were then transferred to individual pots which were moved to outdoor experimental plots. The soil used for potting all plants was John Innes No. 1 (composition: 60% peat and 40% loam). Inflorescences were sampled in May 1999 for GA analysis.

Plants from three populations were used and the F1 generation plants in these populations were sexed by Dr. Anthony Lowe cytogenetically. The term 'population' is used here to refer to a sub-set of F1 generation siblings generated sexually from a single cross. All members of a population are thus genetically different from their fellow population members, and replicates within a single experiment were non-clonal male and female individuals from a single population.

The inflorescences were approximately 2 to 2.5 cm long at the time of sampling. Although at this stage the majority of flowers had not yet opened, close examination of the inflorescences with the unaided eye revealed stamens or stigmas in a few flowers per inflorescence and therefore the sex of the plants could be confirmed prior to sampling. The immature inflorescences were snap frozen in liquid nitrogen and stored at −80°C until analysis. The stem tissue below the inflorescence was excluded in the analyses.

Extraction of GAs. The young inflorescence from a single plant was snap frozen in liquid nitrogen and ground to a fine powder under liquid nitrogen. Results were corrected for the loss of GAs resulting from extraction and purification. To this end 670 Bq of [^{3}H]GA_{20} was added to each sample. Radioactivity recovered was estimated by scintillation counting using a Tri-Carb 2000 liquid scintillation analyzer (United Technologies Packard, Packard Instrument Company, Meriden, USA). Each sample was allowed to extract for 2 hours in 30 ml of 80% methanol (v/v) and the extract was centrifuged at 900 × g, 6°C, for 10 min. The supernatant was reduced to near dryness by rotary evaporation. The sample was resuspended in water to give a total volume of 10 ml and centrifuged as before for 5 min.

The extract was then partially purified on C_{-18} Sep-Pak cartridges. Each cartridge was activated by passing 10 ml of 100% methanol through it and washed with 10 ml of 2 mM acetic acid. The sample was passed through a cartridge and the eluate pas-

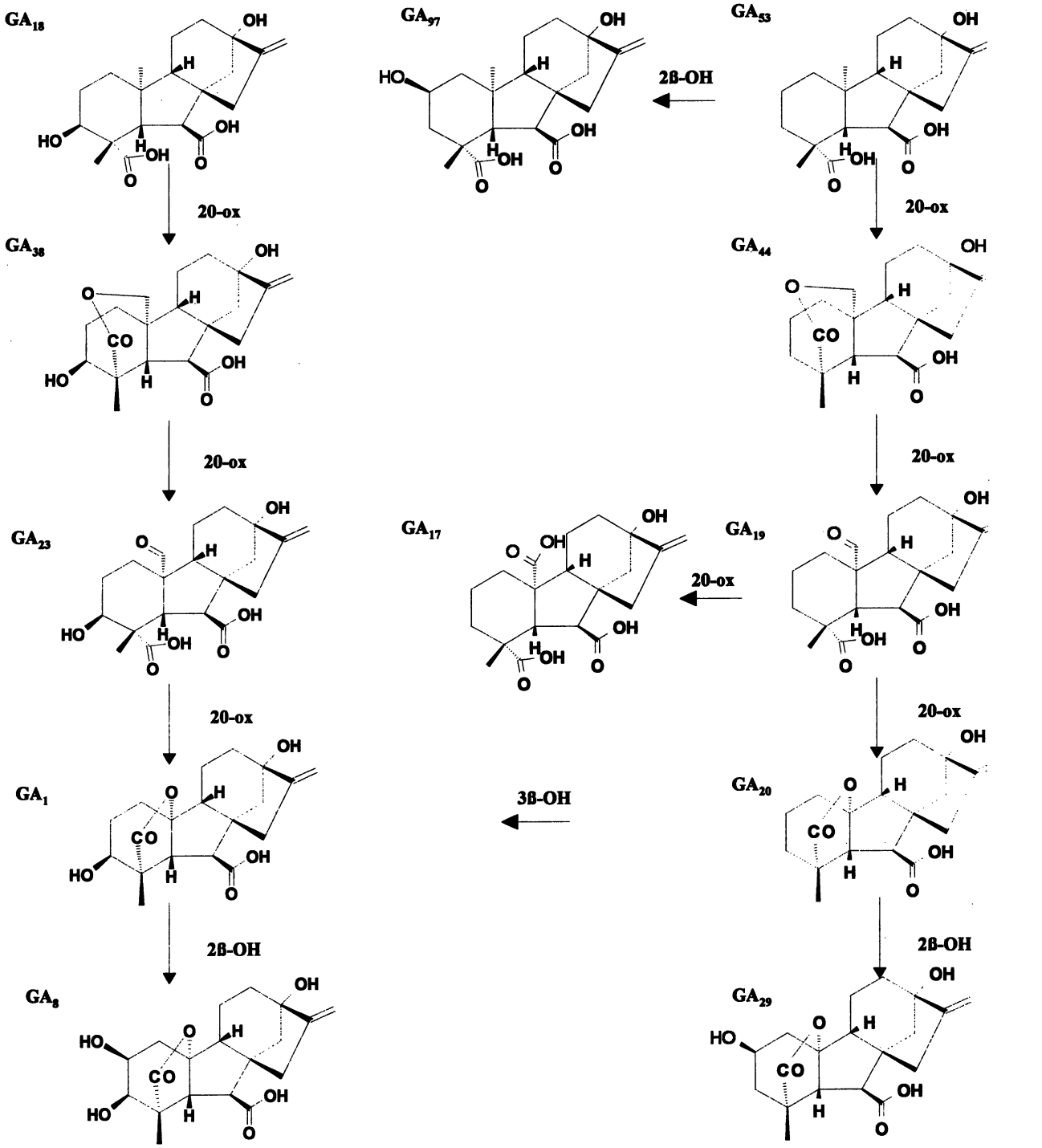

Figure 1. The putative early 3β, 13-hydroxylation pathway (left) and the early 13-hydroxylation pathway (right). All the GAs of the pathways except for GA_{20} and GA_{53} have been previously identified in *R. acetosa* inflorescences using GC-MS or GC-MS/MS (Stokes and others 2003). The spectra for GA_{53} in the inflorescences were contaminated, therefore absolute identification was not possible, but GA_{53} was identified in the leaves. GA_{17} and GA_{97} were only identified in the sample of female inflorescences.

sed to waste. The bound fraction was eluted with 10 ml of 80% of methanol and reduced under vacuum to near dryness. The sample was resuspended in 1 ml of water. For further purification, 20 ml of 10 mM acetic acid was added to 1 g of insoluble polyvinyl polypyrolidone (pVPP) in a centrifuge tube. The

slurry of pVPP was shaken for a few minutes and the denser particles allowed to settle. After 15 min the upper layer containing fine particles of pVPP was decanted to waste. The process was repeated a total of five times. Finally, an aliquot of the plant extract and enough water to create a slurry were added to the activated pVPP sediment. The slurry was shaken on an orbital shaker (R100 Luckham, Sussex, UK) at 1 Hz for 30 min and then centrifuged at 900 × g for 15 min. The supernatant was reduced under vacuum and resuspended in 1 ml of water.

The GAs were separated by reverse-phase HPLC on a 150 mm × 4.6 mm column of 5 μm octadecylsilica (Anachem, Rainin Instruments, Massachusetts, USA) with a SP8700 HPLC solvent delivery system (Spectra Physics, St. Albans, UK) and a SP4100 computing integrator (Spectra Physics, St. Albans, UK). Authentic GAs were detected using a LC 871 UV-Visible detector (Pye Unicam Ltd., Cambridge, UK). The gradient used in the separation was based on a mobile phase that allows detection of authentic GA standards at a UV wavelength of 206 nm (Barendse and others 1980). Authentic GA standards (purchased or a gift of Professor Lewis Mander of the Research School of Chemistry, Australian National University, Canberra, Australia) were used to check retention times. The separation program ran as a gradient from 18% (v/v) methanol/2 mM acetic acid to 60% (v/v) methanol/2 mM acetic acid in 20 min, then isocratically at 60% (v/v) methanol/2 mM acetic acid for 15 min. The column was then washed by running 100% methanol for 15 min. The flow rate was set to deliver 1 ml/min and fractions were collected at 1 min intervals. For each sample, 35 fractions were collected and each fraction was dried in a Univap centrifugal evaporator (Uniscience Ltd., London, UK) set to 37°C. The dried pellets were resuspended in 1 ml of water and the GA content of the individual fractions was analyzed using ELISAs.

ELISA Analysis. The indirect MAC 136 and MAC 182 ELISA protocols used for GA analysis are presented in Huntley and others (2002). The MAC 182 antibody was kindly provided by Dr. Michael Beale, LARS. This antibody was raised against a GA_4-17-KLH conjugate and is highly specific for the 7-oic acid and the 3β-hydroxyl group in GA_4 (Knox and others 1987). As it has lower affinity for other 3β-hydroxylated GAs such as GA_1 (48% cross reactivity relative to GA_4) it was only used in the present study for initial qualitative studies to investigate the range of putative GA types present in *R. acetosa* tissues.

The MAC 136 antibody was used in the primary ELISA employed for quantification in this study. This monoclonal antibody was purchased from Dr. Geoff Butcher of the Babraham Institute, Cambridge, UK. MAC 136 was raised against the GA_1-3-KLH conjugate, for which GA_1 was coupled to KLH through the 3β-hydroxy group. The antibody recognizes groups distant from the site of coupling and the structural features of the GA molecule; essential for recognition by this antibody are the 7-oic acid and the 13-hydroxyl group. The MAC 136 antibody recognizes a wide range of 13-hydroxylated GAs, as the affinity of the antibody for GAs is little affected by the groups present in the A-ring of the GA molecule and it shows similar cross-reactivities for GAs such as GA_{29}, GA_1 and GA_{20} (Knox and others 1987). The ELISA data were not corrected for the cross-reactivities of the individual GAs and values are expressed as GA_{20} equivalents (GA_{20} is the standard compound used for the MAC 136 ELISA) (Huntley and others 2002).

Quantitative Analysis Using GC-SIM

Plant Material. A larger quantity of tissue was required for GC-MS and GC-SIM analysis (0.5 g DW per sample), so the female inflorescences from several plants from a single population were pooled. The same procedure was adopted for the male inflorescences. Stem tissues were excluded from the samples. The inflorescences were snap frozen in liquid nitrogen and the tissues ground to a fine powder. The powdered tissue was freeze-dried and the samples were analyzed at Long Ashton Research Station, Bristol (LARS).

GC-SRM Analysis

The protocols used for extraction and purification of GAs, and derivatization of GAs for analysis by GC-SRM are as described in Croker and others (1990), except that methylation of samples was carried out prior to reverse-phase HPLC. During the extraction procedure 5–10 ng of each of the following deuterated GAs were added: [17-$2H_2$] GA_8, [17-$2H_2$] GA_{29}, [17-$2H_2$] GA_1, [17-$2H_2$] GA_{20}, [17-$2H_2$] GA_{19}, [17-$2H_2$] GA_{44}, [17-$2H_2$] GA_4, [17-$2H_2$] GA_{53}. In addition, 841 Bq each of tritiated GA_1, GA_{20}, GA_{19} and GA_4 were added to account for losses during extraction and purification. The HPLC gradient was as previously published, except that 2 mM acetic acid was omitted because the GAs were methylated prior to separation by HPLC. GAs were analyzed as methyl ester trimethylsilyl ethers using a Thermoquest GCQ mass spectrometer. The samples were injected into a fused silica WCOT BPX5 capillary column (Scientific Glass Engineering) at an oven

Table 1. GAs Found in Significantly Different Levels between the Sexes in Young Inflorescences

GA	GA content of young inflorescences (pmol/g FW)	
	Male	Female
GA_{53}*	156.4 ± 23.1	228.8 ± 22.0
GA_{19}*	278.3 ± 51.2	538.5 ± 92.4
GA_{29}**	363.9 ± 57.1	163.3 ± 27.7
GA_{18}****	386.4 ± 48.8	141.6 + 27.2
Compound A***	48.8 ± 9.3	133.6 ± 21.8

*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.0005$

Values are the mean (± SE) of 11 male and 11 female plants and derived from the pooled data from three populations. For each experiment GAs from a single extraction of the tissues of a single plant were separated in a single HPLC run and each fraction was assayed in duplicate using indirect ELISAs. Data were corrected to account for losses incurred during extraction, purification and separation, but not for cross-reactivity, and are expressed in pmol/g FW as equivalents of GA_{20}, the standard compound in the ELISAs.

temperature of 60°C. Further details are provided in Coles and others (1999). The amounts of GAs in the sample were determined using previously established calibration curves of peak area ratios of the product ion for unlabelled and deuterated GAs plotted against varying molar ratios of the two compounds (Croker and others 1990).

Sex Expression Studies

Plant Material. Plants used for the experiments were vernalized by overwintering them outdoors and in February 2000 they were moved into a heated greenhouse maintained at 25°C. The use of artificial lighting ensured the plants received 16 hours of light.

Flowering Bioassays. The plants were still in the vegetative phase of development and they were each treated twice with 6 ml of one of the following: 10 μM GA_{18}, GA_{38}, GA_{23} and GA_1 with 0.1 ml l^{-1} Tween. Control plants were sprayed with an equal volume of 0.1 ml l^{-1} Tween. After inflorescences were initiated the developing flowers were treated further with 2–3 μl of a 10 μM solution of the relevant GA. When plants entered the reproductive phase the inflorescences were examined with a hand lens (×10).

Results

Quantitative Analysis Using ELISAs

ELISAs allow the analysis of GAs in quantities of tissue of as little as 0.3 g FW. This enables quantification of GAs in a single inflorescence. Figure 2A and B show the spectra of GAs found in the inflorescences of male and female plants, respectively, from a single population, as analyzed by ELISA. The HPLC gradient used in this study gave a good separation of a wide range of 13-hydroxylated GAs and MAC 136 detected all these GAs.

Analysis of the C_{20}-GAs of the early 13-hydroxylation pathway in a single population of plants (Figure 3A) showed that there were significant sex differences in the levels of GA_{53} in the inflorescences ($p < 0.05$). To investigate whether this difference was a general characteristic of *R. acetosa*, GAs in the inflorescences of other populations were analyzed. Table 1 shows the pooled data from three different populations. Inflorescences from female plants were found to have approximately 50% higher levels of GA_{53} than those from male plants. Quantitative analysis using ELISAs also showed that inflorescences from female plants had higher levels of GA_{19} compared to inflorescences from male plants, although this was not significant for the population shown in Figure 3A due to the wide variance in the data. However, when the data from several populations were pooled, females were found to have significantly higher levels of GA_{19} than males (Table 1).

Of the C_{19}-GAs in the early 13-hydroxylation pathway, only GA_{29} was found in significantly different amounts between the sexes ($p < 0.05$) (Figure 3B). This difference was also significant when the data from three populations were pooled, with the inflorescences from males having approximately 2.2 times the GA_{29} content of females ($p < 0.01$).

Comparison of GA levels between the two sexes in the inflorescences from a single population (Figure 3C) showed that there was a significant difference in GA_{18} levels, with males having higher levels than females ($p < 0.05$). Looking at the pooled data

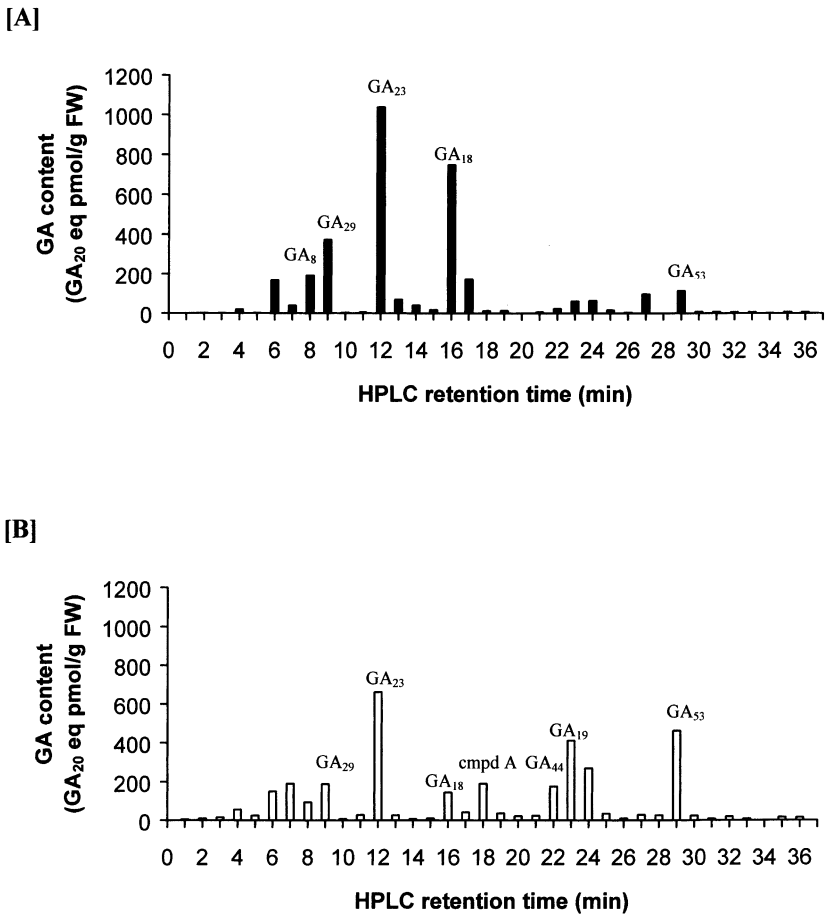

Figure 2. Immunoanalysis of the GA spectrum and content in purified extracts of young inflorescences sampled from a single [A] male and [B] female *R. acetosa* plant. The plants were grown under natural conditions in an experimental plot and inflorescences were collected in late spring. For each experiment GAs from a single extraction of the tissues of a single plant were separated in a single HPLC run and each fraction was assayed in duplicate using indirect ELISAs. Data were corrected to account for losses incurred during extraction, purification and separation, but not for cross-reactivity, and are expressed in pmol/g FW as equivalents of GA_{20}, the compound used as the standard in ELISAs. Identification of immunoassay peaks were carried out by comparison with the retention time of authentic GAs. An unidentified putative GA detected in the samples is referred to as compound A (cmpd A).

from three populations (Table 1), this trend was found to be highly significant with male inflorescences having approximately 2.7 times the GA content of females ($p < 0.0005$).

ELISA data from the GA analysis of inflorescences revealed a putative GA whose retention time on HPLC did not correspond to any available GA standards in published literature and its identity has not been determined by GC-MS. It is referred to as compound A. Female inflorescences had significantly higher levels of this putative GA than males ($p < 0.005$).

Quantitative Analysis Using GC-SRM

Qualitative analysis was carried out first to determine the full complement of GAs in the tissues and this was followed by quantitative analysis using an appropriate suite of deuterated standards. The GC-SRM data confirmed the trends observed for the GAs of the early 13-hydroxylation pathway using ELISAs, with the inflorescences from female plants having higher levels of GA_{53} and GA_{19} than those from males, whereas the inflorescences from male plants had higher levels of GA_{29} than those from females (Table 2).

Due to the lack of deuterated standards, GA_{18} and GA_{97} levels could not be quantified by GC-SRM. GA_{97} could not be quantified using ELISAs because authentic standards were not available for estimation of retention time. However, it was considered important to estimate the levels of these GAs because the ELISA data indicated there were differences between the sexes in the content of GA_{18} in the inflorescences. Qualitative GC-MS data showed that the content of GA_{97} was below the limit of detection in the inflorescences from male plants (Stokes and others 2003). GA_{18} and GA_{97} elute between fractions 18–22 and GA_1 in fractions 16–17. By pooling fractions 16–22, the GA_{18} and GA_{97} content could be quantified using the deuterated GA_1 standard and although absolute GA values could not be measured, values relative to the GA_1 standard were obtained. They indicated that the level of GA_{18} in male inflorescences was approximately nine times that in the females. GA_{97} values were similar to GA_{18} values in the female inflorescence and, again,

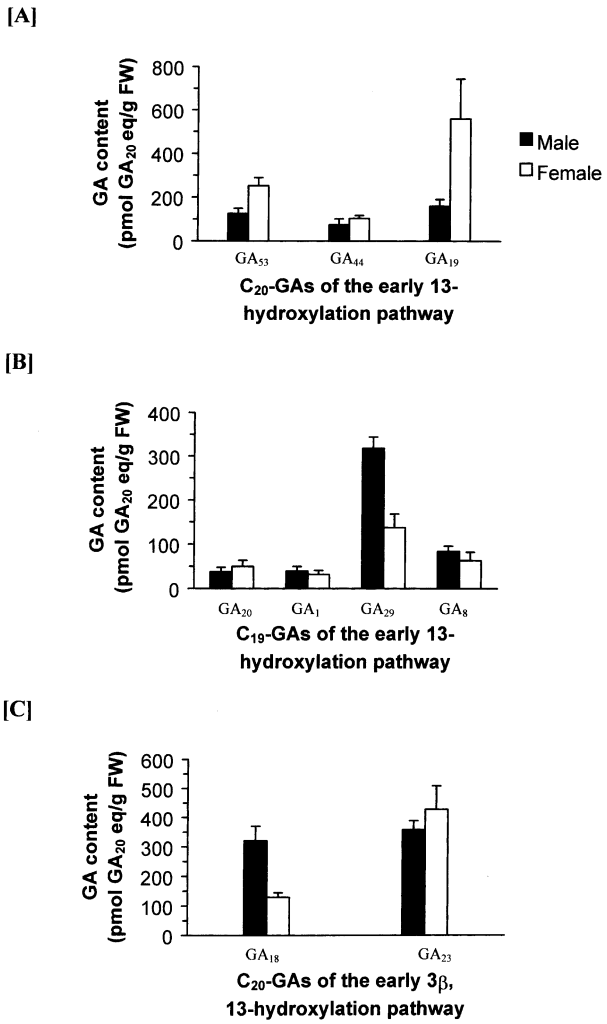

Figure 3. GA content of young inflorescences from male and female plants of a single population. Data refer to the mean (±SE) of four male and four female plants. For each experiment GAs from a single extraction of the tissues of a single plant were separated in a single HPLC run and each fraction was assayed in duplicate using indirect ELISAs. Data were corrected to account for losses incurred during extraction, purification and separation techniques, but not for cross-reactivity, and are expressed in pmol/g FW as equivalents of GA_{20}, the compound used as the standard for the ELISAs. Females had significantly higher levels of GA_{53} ($p < 0.05$) [A] and males significantly higher levels of GA_{18} ($p < 0.05$) [C] and GA_{29} ($p < 0.01$) [B].

GA_{97} was not detected in the male inflorescences (Table 2).

Metabolic Conversions

Using ELISA data, comparisons were made between the sexes of the ratios of consecutive GAs of the early 13-hydroxylation pathway in inflorescences (Table 3). This yields information about metabolic conversions at the time of sampling and contributes towards understanding the source of the difference in the content of certain GAs between the inflorescences of male and female plants. Although females generally had higher levels of GA_{53} than males, this is unlikely to be due to males having higher rates of conversion to GA_{44}, resulting in a higher GA_{44}/GA_{53} ratio, because there was no significant difference between the sexes in their GA_{44}/GA_{53} ratios. Perhaps the higher level of GA_{53} in the female inflorescences is due to higher rates of conversion from a precursor GA to GA_{53} than in the males. The precursor is likely to be either GA_{12} or GA_{53} aldehyde.

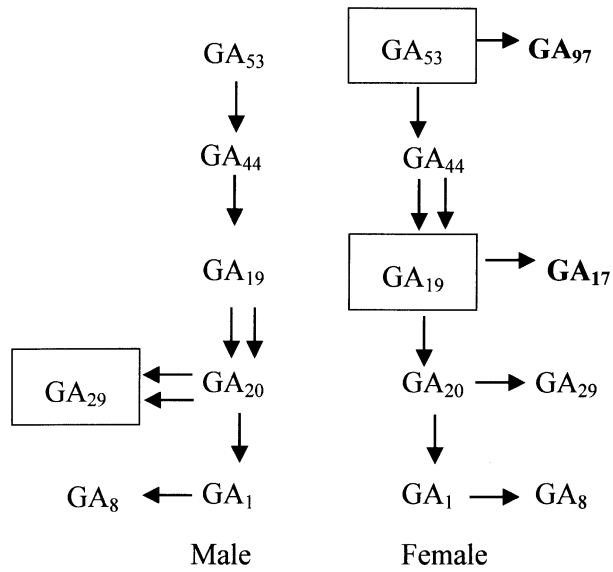

Figure 4. Schematic of sex-differences in the metabolism of GAs of the early 13-hydroxylation pathway in young inflorescences. The scheme is based on qualitative analysis by GC-MS and quantitative analysis by ELISAs and GC-SRM. Double arrows indicate that the ratio of GA conversion at that stage was approximately twice that detected for the other sex. GAs that were only identified in one sex are found to be in bold. GAs that were found to be significantly more abundant in one sex than the other by ELISAs are shown enclosed in a rectangle in the case of the sex in which they were detected at higher levels.

The inflorescences sampled from female plants also had higher levels of GA_{19} than those from males. This was found in both the ELISA and GC-MS quantitative data. It may be due to increased conversion of GA_{44} to GA_{19}, indicated by a higher GA_{19}/GA_{44} ratio in female samples than males, and/or a decreased conversion to GA_{20}, indicated by a low GA_{20}/GA_{19} ratio in females compared to males. The results showed that females did indeed have

Table 2. The GA Content of Young Inflorescences Quantified using GC-SRM

	GA content of young inflorescences (pmol/g FW)	
GA	Male	Female
GA_8	74.0	24.3
GA_{29}	8.6	2.3
GA_1	2.7	1.3
GA_{20}	1.2	2.2
GA_{19}	12.4	96.1
GA_{53}	Contaminated spectrum	Contaminated spectrum
	GA content of young inflorescences (expressed as a ratio relative to the $GA_{1standard}$)	
GA_{18}	4.9	0.5
GA_{97}	Not detected	0.5

Values for each sex are from the analysis of a single sample of tissue pooled from plants of the same population. No deuterated standards for GA_{18} or GA_{97} were available and therefore they were quantified by reference to deuterated GA_1.

Table 3. Ratios between successive GAs of the Early 13-hydroxylation Pathway.

Ratios between GAs	Male	Female
GA_{44}/GA_{53}	0.6 ± 0.1	0.4 ± 0.3
GA_{19}/GA_{44}	4.1 ± 1.1*	8.3 ± 1.6*
GA_{20}/GA_{19}	0.20 ± 0.04*	0.10 ± 0.02*
GA_{29}/GA_{20}	12.2 ± 2.9	5.5 ± 1.5
GA_1/GA_{20}	1.1 ± 0.1*	0.74 ± 0.07*
GA_8/GA_1	3.7 ± 1.0	3.5 ± 1.0

**The ratios that are significantly different between male and female samples ($p < 0.05$). See the legend to Table 1 for a summary of the experimental techniques used.Values are the mean (±SE) of 11 males and 11 females*

significantly higher GA_{19}/GA_{44} ratios ($p < 0.05$) and also that the male inflorescences had a significantly higher ratio of GA_{20}/GA_{19} ($p < 0.05$). An increase in the synthesis of GA_{19} coupled with its reduced conversion to GA_{20} may explain the higher levels of this GA detected in the inflorescences from females compared to males.

In contrast, GA_{29} levels were found to be significantly higher in males than females. No GA_{29} catabolites were detected in the GC-MS analysis. Therefore, as an inactive end-of-the-line GA, the content of GA_{29} is expected to be regulated by its synthesis from GA_{20}. Although the difference is only significant at the 6% level, the average GA_{29}/GA_{20} ratio in males is approximately twice that of females, suggesting a higher level of inactivation of GA_{20} in males than females. Higher levels of the biologically active GA_1 were detected in male inflorescences using GC-SRM than in female inflorescences (Table 2A). Although not statistically significant because of a wide variance in data, this was also detected using ELISAs (Figure 3B). This trend may be the consequence of a higher level of conversion from GA_{20} to GA_1 in male than in female inflorescences. A significantly higher GA_1/GA_{20} ratio was detected in the male than in the female inflorescences ($p < 0.05$) (Table 3). No significant difference was found between the sexes in the GA_8/GA_1, showing that differences in inactivation of GA_1 are unlikely to be a cause of the higher levels of GA_1 in the male inflorescences.

Sex Expression Studies

Male and female plants in the vegetative phase of development were treated exogenously with a range of GAs of the putative early 3β, 13-hydroxylation pathway. When the plants developed inflorescences, a hand lens was used to observe the floral organs in the flowers.

No development of inappropriate sex organs was observed in the floral structures of the young inflorescences compared to control plants. Further applications of the same GAs directly to the developing flowers also did not induce any visible changes in floral structures other than those expected in normal floral development.

Discussion

GA_{97} was identified and semi-quantified in female inflorescences, but not detected in male inflorescences. It is likely that the synthesis of inactive metabolites such as GA_{97} may function as a ''safety valve'' by inactivating precursors when endogenous C_{20}-GA levels are high. This proposal was supported by the results of quantitative ELISA analysis of the GA_{53} content of young inflorescences, in which females were found to have higher levels of this GA than males, despite the diversion of GA_{53} to GA_{97} in females. Mander and others (1996) have also suggested that high levels of 2β-hydroxylated C_{20}-GAs, such as GA_{97}, may reflect a process of inactivation of C_{20} precursor types which may accumulate when the early 13-hydroxylation pathway is limiting synthesis of active GAs, for example, under SD

conditions. R. P. Huntley ('Cytokinins and Gibberellins in Oil Palm Sex Determination', unpublished PhD thesis, University of Cambridge, UK, 1995) and Juntilla and others (1997) have used the ratios of the levels of successive GAs in GA biosynthetic pathways to provide information on the relative rate of metabolic conversions in the pathway. A comparison of GA_{44} and GA_{53} levels from this study (Table 3) shows that the higher levels of GA_{53} in the female inflorescences cannot be attributed to a lower conversion to GA_{44}, but may be the result of greater synthesis from precursor GAs, such as GA_{12} to GA_{53}.

Both GC-SRM and ELISA analysis revealed that another C_{20}-GA, GA_{19}, was one of the most abundant GAs in female inflorescences, but not in males. A comparison of the ratios of GA_{19} to GA_{44} and GA_{20} to GA_{19} in both males and females (Table 3) indicates that the relatively high levels of GA_{19} in the females could be due to a higher rate of synthesis from GA_{44} coupled to a lower rate of conversion to GA_{20}. In plants the formation of GA_{20} from GA_{53} is carried out via increasing oxidation of C-20 by GA_{20}-oxidases (MacMillan 1997). As discussed by Hedden and others (2002), the *Arabidopsis* genome contains potentially five *GA20ox* genes and some of these have been shown to have tissue-specific expression (Phillips and others 1995). It may be speculated that in *R. acetosa* inflorescence tissues, more than one 20-oxidase enzyme is involved in the conversion of GA_{53} to GA_{20}, with different levels of activity in the two sexes. It is pertinent to add that GC-MS analysis revealed the presence of GA_{17} in female, but not male inflorescences (Stokes and others 2003). This is an inactive tricarboxylic acid metabolite of GA_{19} and its synthesis is probably a consequence of the high levels of GA_{19} in female inflorescences.

Looking at compounds further down the early-13 hydroxylation pathway, ELISA data revealed that males had on average 100% more GA_{29}, the C_{19} 2β-hydroxylated metabolite of GA_{20}, than females. Similarly, the GC-SRM data revealed that the inflorescences from males had higher levels of this GA than those from females. Based on ELISA data the ratio of GA_{29} to GA_{20} in males was approximately twice that of females and therefore it is proposed that the higher level of GA_{29} in the males is due to higher rates of inactivation of GA_{20} in males compared to females.

As discussed earlier for the C_{20}-GAs, the conversion of C_{19}-GAs in plants to inactive metabolites by 2β-hydroxylation down-regulates the GA-biosynthesis pathway and lowers levels of active GAs (Hedden and others 2002). In the inflorescences of *R. acetosa*, as summarized in Figure 4, analysis of GAs seems to suggest that males and females utilize this mechanism to different extents at various steps in the early 13-hydroxylation pathway to reduce the levels of precursors of active GAs. Using GC-SRM, the levels of the C_{19}-GAs—GA_1 and in particular the 2β-hydroxylated metabolites GA_8 and GA_{29} —were found to be higher in male inflorescences. Similarly, using ELISAs, significantly higher levels of GA_{29} were also detected in young male inflorescences than females. Therefore, it appears that the conversion from C_{20}- to C_{19}-GAs such as GA_{20} and subsequently from GA_{20} to GA_1 in GA biosynthesis is restricted less in male inflorescences than in females. Thus, a lower rate of conversion of precursor C_{20}-GAs to active types, relative to that of males, may lead to accumulation of these GAs in female inflorescences.

In addition to the well-known GAs of the early 13-hydroxylated pathway, an unidentified putative GA was picked up by the MAC 136 ELISA in extracts of young inflorescences. This implies that it is a 13-hydroxylated GA. As seen in Figure 2B, it elutes from a C18 column in reverse-phase HPLC just later than GA_{18}, but prior to GA_{20}. It is therefore most likely to be a dihydroxy GA. The retention time of this compound did not correspond to any available GAs, and female inflorescences were found to have higher levels of this compound.

Contrary to the trend observed for the C_{20}-GAs—GA_{53} and GA_{19} —of the early-13 hydroxylation pathway, GA_{18}, a C_{20}-GA of the putative early 3β, 13-hydroxylation pathway, was found to be present at higher levels in male inflorescences. Although deuterated standards for quantification of GA_{18} by GC-SRM were unavailable, the content of GA_{18} in inflorescences was estimated relative to the deuterated GA_1 standard and the estimate strongly confirmed the trend observed with ELISA analysis. The precursor (possibly GA_{14}, see Durley and others 1974) to and immediate product (GA_{38}, see MacMillan 1997) of GA_{18} in the early 3β, 13-hydroxylation pathway were not quantified by ELISA or GC-SRM and therefore the specific metabolic steps governing the GA_{18} levels in the inflorescences could not be predicted.

Čulafič (1999) found that treatment of dioecious *Rumex acetosella* with plant growth inhibitors did not result in hermaphrodite plants or a shift in sex. Similarly he found that treatment of plants with GA_3 did not affect their sex. Although GA_3 is widely used in studies because it is the most readily available biologically active GA, it is not known whether this GA occurs endogenously in *R. acetosella*. In this study it was thought to be more physiologically

relevant to test GAs shown to be endogenous in *R. acetosa*. The biological activity of the C_{20}-GAs of the early 3β, 13-hydroxylation pathway have been examined in some plant systems and a general trend was for GA_{38}, with its C-19-C-20 δ-lactone bridge, to show greater activity than GAs with a C-20 methyl group, such as GA_{18} (Reeve and Crozier 1974; Kamiya and others 1991; Stokes and others 2003). Because male *R. acetosa* inflorescences had significantly higher levels of GA_{18} than female inflorescences, it was pertinent to examine whether this and other GAs of the early 3β, 13-hydroxylation pathway were involved in sex determination.

Although any minor promotion of floral primordia may not have been apparent on examination under a hand lens, it was clear that treatment of male and female plants with these GAs did not elicit any obvious changes in the development of the sex organs and there was no major change in the sex of the flowers. Therefore it is suggested that GAs of the early 3β, 13-hydroxylation pathway and possibly GAs in general are not involved in sex determination of *R. acetosa*. It is probable that in this species the differences in GA content between the sexes are a consequence of sex expression. Detailed analytical studies of the GA content of the floral sex organs dissected from the flowers should reveal the distribution of GAs within male and female flowers.

Using analytical techniques such as GC-MS, GC-SRM or ELISAs, several studies on dioecious plants such as Kentucky coffee bean, *Rumex acetosella*, *Mercurialis annua* and *Leucadendron rubrum* have shown qualitative or quantitative differences between the sexes in their content of cytokinins (Hautala and others 1986; Jelič and others 1988; Durand and Durand 1991; De Kock and others 1994). Also using GC-SRM or ELISAs, differences in auxin content have been revealed in the dioecious species *Mercurialis annua* and *Asparagus officinalis* (Hamdi and others 1987; Bracale and others 1991). By contrast, using combined HPLC-ELISAs with antibodies specific for isopentenyladenine-type, zeatin-type and dihydrozeatin-type cytokinins, no qualitative or significant quantitative differences were detected in male and female *R. acetosa* in analyses of the isoprenoid cytokinin content of the inflorescences and upper stem tissues (T. S. Stokes 'Gibberellins and cytokinins in *Rumex acetosa* L.', unpublished PhD thesis, University of Cambridge, UK, 2001). The possible involvement of other plant growth regulators such as ethylene in the sex determination process in *R. acetosa* remains to be investigated.

To the authors' knowledge, although there have been many attempts to change the sex of dioecious plants by exogenous GA application (reviewed by Čulafič 1999; Khryanin 2002), no recent studies have examined the endogenous GA concentration of such plants using modern analytical techniques. However, as this study on the GA content of inflorescence tissues of *R. acetosa* has demonstrated, when the sexes are analyzed separately, qualitative and quantitative differences in their endogenous GA content may be revealed, contributing to a more realistic representation of GA metabolism in the species.

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