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3β,13-Dihydroxylated C_{20} gibberellins from inflorescences of *Rumex acetosa* L.

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Abstract

Using full scan GC–MS a wide range of gibberellins (GAs) was identified in the young inflorescences of the dioecious species *Rumex acetosa* L., consistent with the ubiquitous early 13-hydroxylation pathway in both male and female plants. In addition, *R. acetosa* is the first species in which all three 3β,13-dihydroxylated C_{20}-GAs—GA_{18}, GA_{38} and GA_{23}—have been identified in the same organism, suggesting an early 3β,13-dihydroxylation biosynthesis pathway in this species. Authentic GA_{18}, GA_{38} and GA_{23} were synthesized and their effects and that of GA_1, a GA common to both pathways, on the time to inflorescence emergence was investigated. GA_1 accelerated the emergence of inflorescences in both male and female plants. In addition some evidence for biological activity per se of the C_{20}-GA_{38} was obtained.

Keywords: *Rumex acetosa*; Polygonaceae; Sorrel; Synthesis; GC–MS; Gibberellin; Early 13-hydroxylation pathway; Early 3β,13-hydroxylation pathway; GA_{18}; GA_{38}; GA_{23}

1. Introduction

Rumex acetosa L. (common sorrel) is a dioecious perennial (Korpelainen, 1992). Inflorescences are initiated in spring and early summer and it is during the reproductive phase of development that the phenological differences between the sexes become most evident (Ainsworth et al., 1999). Gibberellins (GAs) in *R. acetosa* shoots have been analysed previously by full-scan GC–MS (Rijnders et al., 1997). GA_{53}, GA_{29}, GA_{19}, GA_4 and GA_1 were identified, which suggested the operation of two biosynthesis pathways in these tissues: the early 13-hydroxylation pathway and the non 13-hydroxylation pathway. In the current paper we report the results from a study using full-scan GC–MS to identify the suite of endogenous GAs in young inflorescences of *R. acetosa*. Unlike the previous study, where the sex of the plants was not specified, the samples from male and female plants were analysed separately to reveal any qualitative differences in their content of GAs.

Since the seminal work of Lang, many studies have demonstrated the involvement of GAs in the floral induction of photoperiod-sensitive species under non-inductive conditions and/or in accelerating flowering under inductive conditions (Michniewicz and Lang, 1962; Wilson et al., 1992; Hisamatsu et al., 1998; Lee et al., 1998). Rosette plants do not show any obvious stem development in the vegetative state, but produce a flowering stem during the reproductive phase of development. The use of GA biosynthesis inhibitors on some rosette plants, such as *Beta vulgaris* (Van Roggen et al., 1998) and *Silene armeria* (Talon and Zeevaart, 1990), has revealed that, although GAs are not involved in floral induction in these species, they are required for stem elongation. In *Rumex acetosa,* the involvement of GAs during the reproductive phase of development has not been previously investigated. However, in a related

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species, *R. acetosella*, exogenous treatment of plants with GA_3 under long-day (LD) conditions advanced the production of 'floral buds' by 70 days from the time of treatment, compared to control plants (Bavrina et al., 1991).

From early biochemical studies to the more recent molecular studies there is persuasive evidence for the role of GAs in floral induction of the facultative LD rosette plant *Arabidopsis thaliana*. Non-vernalizable 'races' of this species flower under both LD and SD conditions, but flowering is considerably accelerated under LD conditions. An early study by Langridge (1957) demonstrated that inflorescence induction was promoted by GA_3 under SD conditions. There is now evidence of at least three flowering pathways in *A. thaliana*: a GA-dependent, a LD and an autonomous pathway (Reeves and Coupland, 2001).

The *ga1-3* mutants in *A. thaliana* are impaired in their synthesis of CPP synthase which catalyses the conversion of GGPP to CPP in early GA biosynthesis. Consequently the GA levels in these mutants is greatly reduced (Sun and Kamiya, 1994). Wilson et al. (1992) found that under continuous light the *ga1–3* mutants initiated flowers, but flowering was delayed compared to wild-type plants, possibly indicating a partial requirement for GAs for flowering under such conditions. However, under non-inductive SD conditions, *ga1-3* mutants did not flower unless treated with exogenous GA_3, therefore showing an absolute requirement for GAs under such conditions.

In *A. thaliana* the transition to flowering is partly dependent on the inflorescence meristem identity gene *LEAFY* (*LFY*). As the plant develops, levels of *LFY* expression in the primordia on the shoot apex increase until a threshold is reached and subsequently floral rather than leaf primordia are developed (Blázquez et al., 1998). Blázquez et al. (1998) found that the failure of the severely GA-deficient mutant *ga1-3* to flower in SD corresponded to the lack of significant upregulation in expression of *LFY* during the three months of the experiment. Constitutive expression of *LFY* in such mutants restored their ability to flower under SD conditions.

Biochemical studies using exogenous applications of a wide range of authentic GAs have revealed that the extent of biological activity of GAs in advancing flowering and/or promoting bolting may depend on the structural features of the GA molecules, as well as the species under investigation (Evans et al., 1990; Mandel et al., 1992). For example, GAs without a hydroxyl group on C-13 but carrying one at C-3, such as GA_4, promote both inflorescence initiation and stem elongation in *Matthiola incana* (Hisamatsu et al., 2000), but in *Lolium temulentum* GAs with a hydroxyl group at C-3 have reduced levels of activity in promoting inflorescence initiation, while the presence of a hydroxyl group on C-13 increased activity (Evans et al., 1990).

Relatively few GAs are considered to be biologically active (Lange, 1998). Treatment of mutants defective in GA biosynthesis with authentic labelled or unlabelled GAs has demonstrated that certain C_{19}-GAs such as GA_1, GA_3, GA_4 and GA_5 have biological activity per se (Zeevaart and Talon, 1992; Spray et al., 1996). However, as suggested by Lange (1998), GAs with relatively low or negligible activity in common bioassays may be physiologically active in some plant species, at specific development stages and/or in certain tissues. Early bioassays have indicated that some 3β,13-hydroxylated C_{20}-GAs may have intrinsic biological activity, that is their activity is not entirely dependent on metabolic conversion to C_{19}-GAs (Reeve and Crozier, 1974). Following the identification of GA_{18}, GA_{38} and GA_{23} in *R. acetosa* tissues by full-scan GC–MS, authentic samples of these GAs were prepared and their biological activity was examined in flowering time studies.

2. Results and discussion

Nine GAs—GA_8, GA_{29}, GA_{17}, GA_{97}, GA_{17}, 16, 17-dihydro-17-hydroxy GA_{53}, GA_{18}, GA_{38} and GA_{23}—were identified for the first time in this species and were detected in inflorescence tissues (Table 1). GA_{17} and GA_{97}, the inactive metabolites of GA_{19} and GA_{53} respectively, were only identified in the sample of inflorescences from female plants. The presence of GA_{18}, GA_{38} and GA_{23} is consistent with the operation of the putative early 3β, 13-dihydroxylation pathway (MacMillan, 1997) in floral tissues, although local accumulation of these GAs from other sites of synthesis cannot be discounted. As reported previously for *R. acetosa* shoot tissues (Rijnders et al., 1997), GA_{19} and GA_1 were found in the inflorescences, although GA_1 was only found in male inflorescences. In addition GA_{20}, previously identified in shoot tissues (Rijnders et al., 1997), was identified in GC–SIM quantitative studies of both male and female inflorescences (data not included), although it was not detected in the qualitative studies. The spectrum of GA_{53} was too contaminated to allow unambiguous identification, but it was confirmed in leaves of male plants. Although GA_4 and GA_9 of the non-early-13 hydroxylation pathway have been reported as present in shoot tissues of *R. acetosa* by Rijnders et al. (1997), they were not detected in the inflorescences of either male or female plants in this study.

GA_{18}, GA_{38} and GA_{23} are 20-carbon GAs that are both 3β and 13 hydroxylated. They were initially identified in the seeds of species such as *Lupinus luteus*, *Wisteria floribunda* and *Phaseolus vulgaris* (Koshimizu et al., 1968b; Fukui et al., 1972; Hiraga et al., 1974). It has been suggested that such GAs may be synthesized via a pathway in which hydroxylation of GA_{12} at C-13 to give GA_{53} and subsequent hydroxylation of C-3 of

Table 1
Endogenous gibberellins identified in *Rumex acetosa* tissues by full scan GC–MS. Identification was based on mass spectra and Kovats retention indices (KRI) of methyl ester trimethylsilyl ether derivatives of GA_S[a]

GA	Source	HPLC fractions	KRI	Major ions (*m*/*z*) with relative abundance (%)
GA_8	Standard		2791	594 (M^+ 100), 579 (3), 565 (2), 535 (7), 504 (4), 448 (24), 379 (6),238 (5), 207 (5), 194 (8)
	Inflorescences (F)	11–13	2790	594 (M^+ 100), 579 (4), 565 (4), 535 (11), 504 (11), 448 (29), 379 (7), 238 (10), 207 (12), 194 (12)
	Inflorescences (M)	11–13	2791	594 (M^+ 100), 579 (4), 565 (2), 535 (11), 504 (4), 448 (20), 379 (6), 238 (4), 207 (8), 194 (8)
GA_{38}	Standard		2991	520 (M^+ 100), 505 (10), 461 (19), 430 (21), 417 (6), 371 (12), 305 (5), 238 (10), 207 (76), 129 (6)
	Inflorescences (F)	11–13	2989	520 (M^+ 46), 505 (1), 461 (9), 430 (13), 417 (11), 371 (16), 305 (nd), 238 (nd), 207 (55), 129 (18)
	Inflorescences (M)	11–13	2988	520 (M^+ 75), 505 (1), 461 (15), 430 (18), 417 (3), 371 (11), 305 (nd), 238 (18), 207 (100), 129 (12)
GA_{29}	Standard		2692	506 (M^+ 100), 491 (9), 477 (11), 447 (6), 389 (11), 375 (15), 303 (20), 235 (10), 207 (38), 193 (7)
	Inflorescences (F)	14–15	2690	weak spectrum 506 (M^+ 100), 491 (nd), 477 (8), 447 (nd), 389 (nd), 375 (4), 303 (19), 235 (22), 207 (23), 193 (11)
	Inflorescences (M)	14–15	2693	506 (M^+ 100), 491 (11), 477 (8), 447 (17), 389 (16), 375(7), 303 (26), 235 (4), 207 (21), 193 (7)
GA_1	Standard		2679	506 (M^+ 100), 491 (9), 448 (18), 377 (12), 376 (14), 313 (9), 305 (5), 208 (8)
	Inflorescences (F)	16–17		Not detected
	Inflorescences (M)	16–17	2678	506 (M^+ 100), 491 (7), 448 (31), 377 (27), 376 (32), 313 (31), 305 (15), 208 (6)
GA_{18}	Standard		2654	536 (M^+ 71), 521 (23), 504 (49), 477 (42), 375 (29), 347 (9), 319 (21), 269 (17), 239 (13), 238 (13), 208 (100)
	Inflorescences (F)	18–22		Not analysed
	Inflorescences (M)	18–22	2656	536 (M^+ 65), 521 (17), 504 (30), 477 (51), 375 (22), 347 (11), 319 (17), 269 (25), 239 (27), 238 (14), 208 (100)
GA_{97}	Standard		2692	536 (M^+15), 504 (6), 477 (10), 446 (4), 387 (8), 371 (9), 327 (12), 239 (46), 208 (100), 207 (84), 179 (25)
	Inflorescences (F)	18–22		536 (M^+42), 504 (21), 477 (33), 446 (11), 387 (20), 371 (20), 327 (35), 239 (10), 208 (100), 207 (66), 179 (18)
	Inflorescences (M)	18–22	2695	Not detected
GA_{19}	Standard		2667	462 (M^+3), 434 (100), 402 (53), 375 (50), 374 (95), 345 (46), 285 (15), 239 (35), 208 (3), 193 (13)
	Inflorescences (F)	28–30	2669	462 (M^+ 5), 434 (100), 402 (60), 375 (42), 374 (95), 345 (27), 285 (40), 239 (15), 208 (29), 193 (19)
	Inflorescences (M)	28–30	2670	462 (M^+ 7), 434 (100), 402 (60), 375 (46), 374 (91), 345 (32), 285 (27), 239 (22), 208 (22), 193 (nd)
16, 17–Dihydro-17-hydroxy GA_{53}	Library spectrum			538 (M^+ 7), 448 (10), 407 (54), 389 (8), 375 (100), 347 (24), 297 (19), 241 (13), 207 (17), 181 (19)
	Inflorescences (F)	28–30	2774	538 (M^+ 9), 448 (12), 407 (35), 389 (7), 375 (100), 347 (22), 297 (8), 241 (7), 207 (5), 181 (8)
	Inflorescences (M)	28–30	2776	538 (M^+ 8), 448 (11), 407 (33), 389 (4), 375 (100), 347 (27), 297 (10), 241 (8), 207 (2), 181 (13)
GA_{17}	Library spectrum			492 (M^+ 43), 460 (23), 433 (26), 432 (15), 401 (10), 373 (23), 372 (14), 208 (100), 207 (96)
	Inflorescences (F)	31–33	637	492 (M^+ 30), 460 (64), 433 (18), 432 (46), 401 (15), 373 (20), 372 (26), 208 (100), 207 (38)
	Inflorescences (M)	31–33		Not detected
GA_{53}	Standard		2551	448 (M^+ 74), 433 (18), 416 (49), 389 (56), 357 (12), 251 (13), 241 (34), 208 (100), 193 (15), 181 (31)
	Inflorescences (F)	34–37		Contaminated spectrum
	Inflorescences (M)	34–37		Contaminated spectrum
	Leaves (M)	34–37	2554	448 (M^+ 31), 433 (7), 416 (15), 389 (21), 357 (9), 251 (5), 241 (14), 208 (38), 193 (5), 181 (19)
GA_{23}*	Standard		2677	522 (78), 493 (46), 490 (81), 462 (48), 447 (16), 400 (38), 373 (36), 372 (57), 346 (38), 318 (50)
	Inflorescences pooled (F + M)	12–13	2679	522 (72), 493 (47), 490 (27), 462 (29), 447 (8), 400 (12), 373 (nd), 372 (8), 346 (8), 318 (58)

[a] GA_{23} was identified by GC–MS/MS. Its retention time on HPLC is not comparable with those of other GAs as it was separated on the HPLC as a free acid, not a methylated GA, using a different HPLC system and elution conditions. Abbreviations: not detected (nd), female (F), male (M).

GA_{53} occurred early, the so-called early 3β, 13-hydroxylation pathway (Sponsel, 1995; MacMillan, 1997). Although the possibility of the pathway has been demonstrated in *Pisum sativum* seedlings using *in vivo* radiolabelled feeding studies (Durley et al., 1974), none of the 20-carbon GAs of the pathway have yet been reported in this species. Based on the results of radiolabelled feeding studies and bioassays (Fukui et al., 1972), Durley et al. (1974) proposed that the following conversions occur in the putative early 3β,13-dihydroxylation pathway. Successive oxidation of C-20 in GA_{18} gives initially the δ-lactone GA_{38} and subsequently the aldehyde GA_{23}. Further oxidation of C-20 in GA_{23} leads to the formation of the γ-lactone GA_1 or the tricarboxylic acid GA_{28} (see Fig. 1). To the present authors' knowledge *R. acetosa* is the first species in which three successive 20-carbon GAs—GA_{18}, GA_{38} and GA_{23}—of this potential pathway have been identified together, suggesting that the pathway may be operational in *R. acetosa*.

Although the metabolism of GAs has been researched for decades, it is evident that research on certain aspects of GA biosynthesis is still incomplete. In his review paper MacMillan (1997) showed that some of the stages of the putative early 3β,13-hydroxylation pathway await confirmation by metabolic studies. As the only species in which three consecutive GAs of the pathway have been endogenously identified by GC–MS, *R. acetosa* may be a useful system in which to investigate the metabolic conversions involved in this pathway. In

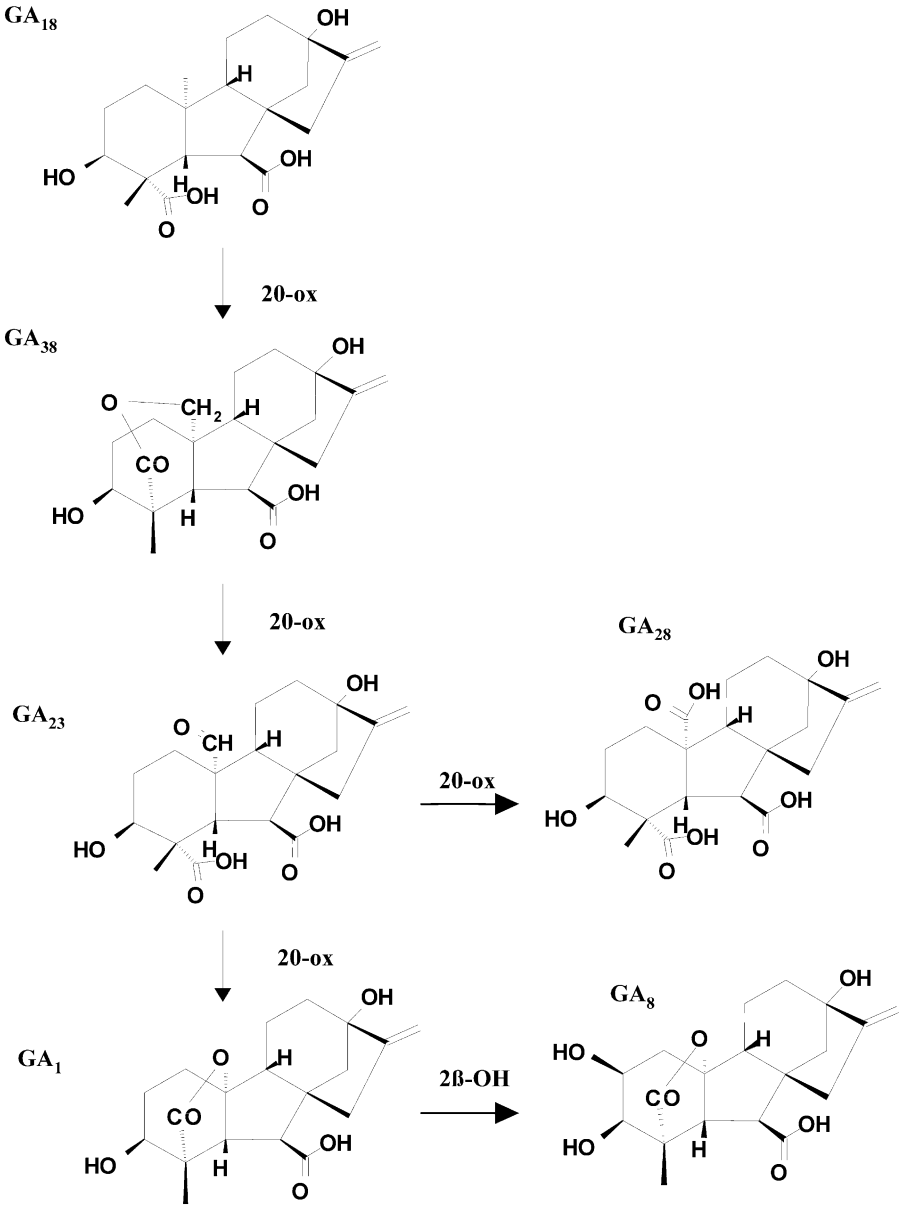

Fig. 1. The putative early 3β,13-hydroxylation pathway. The pathway illustrates the conversions from GA_{18} to GA_1 and its inactive metabolite GA_8. The GA conversions are putatively catalysed by the enzymes 20-oxidase (20-ox) and 2β-hydroxylase (2β-OH).

other species such as *Cucurbita maxima*, although GA_{38} and GA_{23} have been identified endogenously (Lange et al., 1993), the lack of identification of GA_{18} may be because this GA is present at levels too low for current detection techniques. However, it is equally likely that the putative early 3β,13-hydroxylation pathway is not operational in this species. GA_{38} and GA_{23} may be synthesized by 3β-hydroxylation of the 13-hydroxylated GA_{44} and GA_{19} respectively (MacMillan, 1997).

The biological activities of the C_{20}-GAs of the putative early 3β,13-hydroxylation pathway have not been previously investigated in flowering time studies. Fig. 2A shows the typical effects of a range of 3β,13-dihydroxylated GAs and a GA-biosynthesis inhibitor on the time to visible emergence of the inflorescence shoot for male clones ♂A. GA_{18} and GA_{23} had no detectable effects on the time to emergence of the inflorescence shoot in treated compared to the control plants. GA_{38} had a slight promotive effect, giving a mean time to inflorescence shoot emergence of 16 days compared to 20 days in the control plants. GA_1 strongly promoted early emergence by approximately one week compared to control plants. In contrast, paclobutrazol treatment retarded the appearance of the inflorescence shoot by an average of 2 weeks compared to control plants.

A similar trend was observed using female clones ♀B (Fig. 2B). Although the effect was not statistically significant, inflorescence shoots were observed approximately one week later in GA_{23}-treated plants compared to controls. GA_{18} once again had no observed effect on the time to emergence of the inflorescence shoot, while GA_{38} slightly promoted development of the shoot by approximately 4 days. Treatment with GA_1 brought forward the emergence of the inflorescence shoot by approximately 13 days compared to control plants. In contrast, treatment with paclobutrazol retarded the development of the shoot by approximately 25 days compared to control plants.

In studies on other vegetative clones (♂C and ♀D) GA_{38} had a slight promotive effect on inflorescence emergence in the males and inflorescences were detected approximately 2 days earlier in treated compared to control plants. However it had a negligible effect on females (Fig. 2C and D). Again GA_1 showed the greatest biological activity, advancing inflorescence emergence by 6 days in male plants and by 3 days in females compared to control plants.

When data were pooled, irrespective of the clonal populations or the sex of the clones, GA_1 advanced the time to emergence of the inflorescence shoot by an average of 9 days and this was highly significant (Table 2) ($P<0.0001$). A promotive effect on inflorescence emergence was also detected in GA_{38}-treated plants compared to control plants ($P<0.05$). When the data for male and female plants are pooled, the retardation by paclobutrazol of approximately 19 days compared to control plants is also significant ($P<0.05$). Elongation of the inflorescence stem was considerably inhibited in paclobutrazol-treated plants (data not included) and flowering was always positively associated with stem elongation. Similarly in GA_1- and GA_{38}-treated plants there was no indication in any plants of stem growth without inflorescence development.

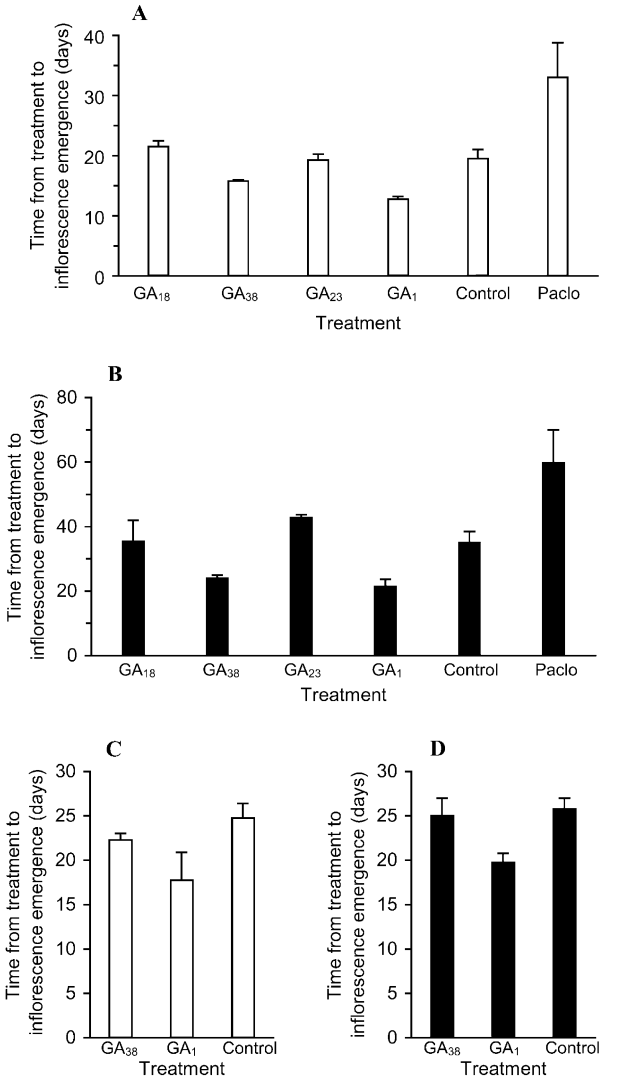

Fig. 2. Time to emergence of the primary inflorescence of males (A and C) and females (B and D) treated with GAs or the GA-biosynthesis inhibitor paclobutrazol (Paclo). Each graph shows data from vegetative clones of a single individual. Plants were vegetatively propagated in September and overwintered outdoors. They were moved into an unheated greenhouse in February the following year. Plants were treated with approximately 6 ml of a 10 μM solution of a single GA (GA_{18}, GA_{38}, GA_{23} or GA_1) applied as a spray with 0.01% Tween, or 50 ml of 0.1 mM paclobutrazol applied as a soil drench. Control plants were treated with 0.01% Tween. After treatment plants were observed daily until a bulbous inflorescence shoot was detected with the unaided eye. Data for each treatment or control show the mean (±SE) of four males and four females, except for the GA_{38}-treated female plants (B) where the mean of three plants is shown. One diseased plant was excluded; it eventually flowered 51 days after treatment.

Table 2
Effects of exogenously applied GAs on days to inflorescence shoot emergence following treatment

Treatment	Days to visible inflorescence initiation
GA_1	19.0±1.1
Control	28.2±1.6
GA_{38}	22.2±1.1
Control	26.1±1.5
Paclobutrazol	46.4±7.4
Control	27.3±3.4

Data shown are the means (±SE) of plants clonally propagated from several individuals and are irrespective of the sex of the plants. GA_1 significantly advanced flowering in plants compared to control plants ($P<0.0001$, $n=23$), as did GA_{38} ($P<0.05$, $n=17$) while paclobutrazol significantly retarded flowering ($P<0.05$, $n=8$). Samples were analysed using two-tailed student *t*-tests.

Treatment with paclobutrazol retarded inflorescence emergence, tentatively indicating a requirement for a threshold level of GAs for the normal development of the inflorescence shoot. GA_1 is primarily implicated in stem elongation. It is not known whether the early emergence of the inflorescences in plants treated with GA_1, 'emergence' being recorded as the time at which the inflorescence shoot was perceptible to the unaided eye, is due to the growth-promoting activities of GA_1 on the inflorescence shoot, or is due to GA_1 acting as a florigenic signal and promoting the vegetative to reproductive phase transition. These considerations may also apply to GA_{38} since the conditions under which the experiments were carried out were conducive to the eventual flowering of all plants, male and female.

Of the C_{20}-GAs of the putative early 3β,13-dihydroxylation pathway, only GA_{38} showed any evidence of activity in advancing the emergence of the inflorescences. A survey of the relative activities of GAs in a range of bioassays found that the activities of the early 3β,13-hydroxylated GAs depended on the plant system in which they were assayed, but GA_{38} showed some activity in four of the five bioassays investigated (Reeve and Crozier, 1974). For example, in the cucumber hypocotyl bioassay, GA_{38} was moderately active and GA_{18} and GA_{23} were inactive, while in the dwarf pea assay, GA_{38} had high activity, with GA_{18} and GA_{23} proving only moderately active. Biochemical studies on the chemical groups that confer activity found that the presence of a C-19–C-20 δ-lactone bridge (as found in GA_{38}) or aldehyde group (as for GA_{23}) resulted in GAs with higher activity than those possessing a C-20 methyl group (as found in GA_{18}) (Hoad, 1983). However, it was not known whether the activity of these GAs was intrinsic, or due to their conversion to other biologically active GAs (Kamiya et al., 1991). Bearing in mind its position in the early 3β,13-dihydroxylation pathway (Fig. 1) it was not unexpected that GA_{38} was more active than GA_{18} in *R. acetosa*. However, the lack of activity of GA_{23} is surprising. If the activity of GA_{38} is due to its eventual conversion to GA_1 it must be first metabolised to GA_{23}. This would suggest that GA_{23} would be at least as active as GA_{38}. However, it is possible that in these studies the uptake and translocation of applied GA_{38} was more effective than GA_{23}. When deuterated or radiolabelled GA_{18}, GA_{38} and GA_{23} become available, feeding with these labelled GAs should reveal whether conversion to labelled GA_1 occurs in *R. acetosa* tissues and whether the putative early 3β,13-hydroxylation pathway occurs in this species.

There is evidence that some GAs δ-lactones can be oxidised in vegetative tissues (Gilmour et al., 1986; Kobayashi et al., 1996). For example a radiolabelled feeding study has demonstrated that the δ-lactone GA_{15} may be oxidized at C-20 in maize seedlings to yield a range of products including 20-carbon and 19-carbon GAs of the early 13-hydroxylation pathway (Davis et al., 1999). However, it has been previously suggested (Reeve and Crozier, 1974; Kamiya et al., 1991) that GA_{38} may have activity per se because the δ-lactone ring in the molecule has functional similarity to the γ-lactone ring of GA_1. If this is so, it is interesting as it indicates intrinsic biological activity in a C_{20}-GA. The findings of early studies which suggested possible activity of C_{20}-GAs have been neglected, perhaps due to the difficulty in obtaining relevant radiolabelled GAs. However, as this study has also indicated, this is an area which warrants further investigation.

3. Experimental

3.1. General experimental procedures

Inorganic chemicals were from Sigma Chemical Co., Poole, UK, unless stated otherwise. The solvents used were of the highest purity: HPLC grade from BDH Chemicals Ltd., Poole, UK. Water used in all solutions was 'polished water' from an Elga system. Infrared spectra were recorded on a Perkin-Elmer 1800 Fourier Transform Infrared spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 instrument. Chemical shifts are reported as values in parts per million. For proton spectra recorded in chloroform, the residual peak of $CHCl_3$ was used as the internal reference (7.26 ppm) for ^1H NMR spectra, while the central peak of $CDCl_3$ (77.0 ppm) was used as the reference for ^{13}C spectra. EI mass spectra (EIMS, 70 eV) were recorded on a VG Fisons Autospec M mass spectrometer. Flash chromatography was carried out on a Merck Kieselgel 60.

3.2. GA analysis

The plants used for analysis were F1 generation progeny of a single cross of native *R. acetosa* plants collected from coastal sites in Cornwall, UK, by Dr. Anthony Lowe, University of Cambridge. The F1 generation plants were grown in the Cambridge University Botanic Garden in outdoor experimental plots. The plants were sexed by Dr. Anthony Lowe using cytogenetic studies and subsequently the sex of plants was confirmed by morphological observation of sex organs in flowers. Analysis was carried out on young inflorescences approximately 2–2.5 cm in length. Inflorescence stem tissues were not included in the analysis. At the time of sampling the majority of flowers that composed the male inflorescences were pre-anthesis and the majority of flowers that composed the female inflorescences were not yet open to reveal the styles.

The inflorescences were snap frozen in liquid nitrogen and ground to a powder in liquid nitrogen. They were freeze dried and stored at −20 °C until analysis. For each sample 0.5 g DW of tissues was used for analysis. The protocols used for extraction and purification of GAs, and derivatisation of GAs for analysis by GC–MS are described in Croker et al. (1990), except that methylation of samples was carried out prior to reverse-phase HPLC. The composition of the gradient used for HPLC was as previously published, except that 2 mM acetic acid was omitted because the GAs were methylated. GAs were analysed as methyl ester trimethylsilyl ethers by full-scan GC–MS using a Finnigan GCQ mass spectrometer, as described in Coles et al. (1999). GAs were identified by comparison of sample KRI and mass spectra with those of authentic standards or library spectra (Hedden, 1986).

Although GA_{23} was tentatively identified by initial qualitative GC–MS following this procedure, the spectra were contaminated. Therefore a different protocol, which is outlined below, was followed for the extraction and purification of samples and this enabled confirmation of the presence of GA_{23}.

GA_{23} was analysed in samples which had been extracted and purified using the following protocols. The young inflorescences from several plants were snap frozen in liquid nitrogen. Each sample (1–3 g FW) was ground to a fine powder using liquid nitrogen. The samples were allowed to extract for 2 h 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 (approximately 500 μl) by rotary evaporation.The sample was resuspended in approximately 9.5 ml of water and centrifuged as before for 5 min.

The extract was then partially purified on C-18 Sep-Pak cartridges, which were activated by passing 10 ml of 100% methanol through them and washed with 10 ml of 2 mM acetic acid. The sample was passed through the cartridge and the eluate passed to waste. Lastly, 10 ml of 80% of methanol was used to elute the bound fraction and this was reduced under vacuum to near dryness. The sample was resuspended in 1 ml of water. The sample was purified further by shaking it in a column containing a slurry of polyvinyl polypyrrolidone on an orbital shaker (R100 Luckham, Sussex, UK) at 1 Hz for 30 min and centrifuged at 900 × *g* for 15 min. The supernatant was reduced under vacuum and resuspended in 1 ml of water.

The GAs were separated using reverse-phase HPLC using a 150 mm × 4.6 mm, 5 μm octadecylsilica column (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 mobile phase used in the separation was based on a gradient that allows detection of authentic GA standards at UV wavelengths at 206 nm (Barendse, 1980). The gradient ran 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 the fraction collector was set to collect fractions at 1 min intervals.

The fractions which corresponded to the retention time of authentic $GA_{23,}$ resulting from the separation of several inflorescence samples were pooled and the combined fractions were taken to dryness. The sample was taken up in 2 ml of methanol and methylated, trimethylsilylated and analysed using GC–MS according to protocols in Croker et al. (1990) and Coles et al. (1999). GA_{23} was confirmed with MS/MS using 552 as the parent ion.

3.3. Synthesis of GA_{18}, GA_{38} and GA_{23}

GA_{23} methyl ester has been prepared from gibberellic acid (GA_3) *via* the 3,13-bis(tetrahydropyranyl) ether (“bis THP”) (Jiang and Pan, 1989) following procedures reported earlier for the synthesis of GA_{36} (Dawe et al., 1985). A repeat of the GA_{23}-Me synthesis, followed by alkaline hydrolysis of the bis THP–GA_{23} ester, then removal of the THP ether protecting groups afforded the parent GA_{23}, in fair yield. Because of equilibrium between the cyclic lactol and aldehyde forms, GA_{23} affords poor NMR spectra and was therefore additionally characterised as its dimethyl ester. GA_{38} was prepared by sodium borohydride reduction of GA_{23} (Koshimizu et al., 1968a), while Wolff-Kishner reduction (Mander et al., 1996) of the bis THP ether of GA_{23} followed by hydrolysis of the THP ether functions furnished GA_{18}.

3.3.1. ent-3α,13,20,20-Tetrahydroxy-gibberell-16-ene-7,19-dioic acid 19,20-lactone (GA_{23})

A stirred solution of bis THP dimethyl ester (88 mg) in MeOH (1.0 ml) was treated with 2M NaOH (5.0 ml) and heated (bath temperature 125 °C) for 15 h. Acidification and extraction into EtOAc, fractionation and chromatography (EtOAc/hexane/AcOH, 2:1:01) on silica gel afforded the corresponding dicarboxylic acid (58 mg) and 7-methy ester (12 mg). Dowex resin (19 mg, H^+ form) was added to a solution of the diacid in MeOH (2.5 ml) and water (0.5 ml), and heated under reflux for 40 min. Extraction into EtOAc followed by drying over Na_2SO_4 then chromatography over silica gel (EtOAc/MeOH/AcOH, 6:0.1:0.05) afforded GA_{23} (25 mg) m.p. 184–190 °C [lit. 185–189 °C (Koshimizu et al., 1968a)]. IR 3400–3100, 2500 (br), 1720, 1703, 1673 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 0.97 (3H, *s*, H18), 2.02 (1H, *d*, partly obscured, H5), 2.02 (1H, *d*, $J = 10.8$Hz), 3.45 (1H, br *s*, H3), 4.63 (1H, *d*, $J = 3.0$ Hz, H17), 4.69 (2H, OH), 4.91 (1H, *d*, $J = 3.0$ Hz, H′17), 5.38 (very br envelope, H20). Dimethyl ester: ^1H NMR (300 MHz, $CDCl_3$): δ 1.21 (3H, *s*, H18), 2.76 (1H, *d*, $J = 12.6$Hz, H5), 3.66 (3H, *s*, OMe), 3.74 (3H, *s*, OMe), 3.88 (1H, *d*, $J = 12.6$Hz, H6), 4.11 (1H, br *s*, H3), 4.93 (1H, *d*, $J = 3.0$ Hz, H17), 5.18 (1H, *d*, $J = 3.0$ Hz, H′17), 9.70 (1H, *s*, H20).

3.3.2. ent-3α,13,20-Trihydroxy-gibberell-16-ene-7,19-dioic Acid 19,20-Lactone (GA_{38})

GA_{38} was prepared by $NaBH_4$ reduction of GA_{23} according to the procedure of Koshimizu et al. (1968a), affording the desired product, m.p. 240–242 °C (lit. 237–239 °C) in 35% yield. ^1H NMR (300 MHz, $CDCl_3$): δ 1.12 (3H, *s*, H18), 2.60, 2.61 (2×1H, 2×ABd, $J = 12.9$ Hz, H6, H5), 3.59 (1H, br *s*, H3), 4.02 (1H, *d*, $J_{gem} = 12.3$ Hz, 20-pro-S-H), 4.36 (1H, *d*, $J_{gem} = 12.3$ *Hz*, 20-pro-R-H), 4.78 (1H, *s*, H17), 5.08 (1H, *d*, $J = 2.4$ Hz, H′17).

3.3.3. ent-3α,13-Dihydroxygibberell-16-ene-7,19-dioic acid) (GA_{18})

Hydrazine hydrate (0.48 ml) was added to a stirred solution of GA_{23} bis THP ether (79 mg) in ethanediol (4 ml) and the reaction mixture heated at 120 °C for 1 h 50 min. Sodium hydroxide pellets (1.7 g) were added and the temperature maintained at 120 °C for 3 h, at which time a precipitate began to form. After a further 30 min the temperature was raised to 175 °C and the reaction maintained at 175–180 °C for 17 h. The cooled mixture was diluted with EtOAc/20% 2-butanol (50 ml) and was acidified to pH 2–3 with 2N HCl. The layers were separated and the aqueous phase was extracted with the EtOAc/2-butanol mixture (2×20 ml). The combined organic phases were washed with brine until the washings were pH 4. The organic phase was dried over sodium sulfate, filtered and the solvent removed under reduced pressure. Chromatography on silica gel (hexane/EtOAc/HOAc, 1:2:0.05) afforded a mixture of 16-ene (major) and 15-ene (minor) acids (46 mg, 58% yield). Dowex 50W-X2 resin (H^+ form) (16 mg) was added to a solution of the crude GA_{18} bis THP ether (46 mg, 0.20 mmol) in methanol (2.5ml) and water (0.50 ml). The reaction was then heated to 60 °C for 1 h 20 min, then at 70 °C for 20 min. The cooled solution was diluted with EtOAc (20 ml) and filtered through Celite, NaOAc added (to pH 6). The MeOH was removed under a vacuum. Then the residue acidified with H_3PO_4 (10%, 1 ml) and diluted with ice cold EtOAc/20% 2-butanol (20 ml). The layers were separated and the aqueous phase was extracted with EtOAc/20% 2-butanol (2×10 ml). The combined organic phases were washed to pH = 4 with ice cold brine. The combined organic phases were dried over sodium sulfate, filtered and the solvent removed in vacuo. Chromatography on silica gel (hexane/EtOAc/AcOH, 1:1:0.05–3:1:0.05) yielded GA_{18} (24 mg) as white crystals, m.p. 239–242 °C [lit. 240–242 °C (Koshimizu et al., 1966)]. IR 3400–3100, 2500 (br), 1703, 1673 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$-CD3OD, 4:1) δ 0.77 (3H, *s*, H20), 1.18 (3H, *s*, H18), 0.80–2.30 (18H, m), 2.19 (1H, *d*, $J = 12.6$ Hz, H5), 3.32 (1H, *d*, $J = 12.6$ Hz, H6), 3.98 (1H, br *s*, H3), 4.84 (1H, br *s*, H17), 5.04 (1H, br *s*, H′17). ^{13}C NMR (75 MHz, CD_3OD) δ 14.2 (C20), 18.1 (C11), 23.3 (C18), 26.6 (C1), 33.3 (C3), 38.8 (C12), 42.9 (C10), 43.8, 44.5, 47.7, 48.1 (C4, C8, C14, C15), 47), 50.1 (C6), 50.3 (C5), 55.9 (C9), 70.6 (C3), 78.2 (C13), 105.7 (C17), 155.6 (C16), 178.2, 179.8 (–CO_2H).

3.4. Bioassays

To minimise natural variations in flowering times plants used for each of the flowering experiments were genetically identical individuals. Plants that were vegetatively propagated from the same individual and therefore were genetically identical are referred to as vegetative clones. The following notation is used to refer to plants: clones of a male individual 'A' are referred to as $♂^A$ and clones raised from a female individual 'B' are referred to as $♀^B$ and so on.

In October, two mature male (A and C) and two mature female plants (B and D), that were F1 generation siblings, were divided longitudinally through the base of the plant into sections which comprised roots, leaves and apical tissues. These were potted individually in John Innes No. 1 (composition: 60% peat and 40% loam). The plants were kept in an unheated greenhouse where they were watered every day until new leaves were produced. The plants were then moved outdoors to experimental plots to undergo vernalization during the winter months.

In February the following year the plants were moved indoors to an unheated greenhouse. The plants were still in the vegetative phase of development. Lighting was

natural in the greenhouse and the plants experienced lengthening photoperiods with the onset of spring. The plants treated with a GA were sprayed on two occasions, 1 week apart. Plants received the first treatment on the day they were moved indoors into the greenhouse and they were treated with one of the following: GA_{18}, GA_{38}, GA_{23} and GA_1. Each plant received a total of approximately 6 ml of 10 μM GA with 0.1 ml l^{-1} Tween, sprayed on the leaves. Control plants were sprayed with an equal volume of 0.1 ml l^{-1} Tween. Plants treated with paclobutrazol received one treatment on the day the plants were moved indoors. Paclobutrazol was applied as a soil drench at a concentration of 0.1 mM with one application of 50 ml per plant.

Plants were monitored on a daily basis and the time from the first treatment of plants to the emergence of the first inflorescence shoot was recorded. Plants were considered to have developed inflorescences when it was evident from the slightly bulbous appearance of the young shoot that the enveloping sheath-like tissue did not contain only leaf primordia, but also enclosed the young inflorescence (Stokes, 2001). In preliminary studies dissection of similar shoots confirmed that a developing inflorescence was enclosed. However, in subsequent non-destructive experiments the presumed young inflorescence were tagged for confirmation at a later stage of development when the inflorescence bearing the flowers was revealed. Each plant could bear several inflorescence stems, but all experiments were carried out on the first inflorescence developed. Data from the flowering experiments were analysed using two-tailed student *t*-tests.

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