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GIBBERELLINS IN INTERNODES AND EARS OF WHEAT CONTAINING DIFFERENT DWARFING ALLELES

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; gibberellins; internodes; ears; quantification; *Rht* dwarfing genes.

Abstract—Gibberellins (GAs) belonging to the main early 13-hydroxylation GA pathway (GA_1, GA_3, GA_8, GA_{17}, GA_{19}, GA_{20}, GA_{29}, GA_{44} and GA_{53}) were identified by full-scan GC-mass spectrometry in rapidly expanding stem internodes and young ears of wheat. In addition, GA_{97} (2β-hydroxy GA_{53}) and GA_{98} (2β-hydroxy GA_{44}) were detected in reproductive, but not vegetative, tissues. Conversely, some members of the non-13-hydroxylation GA pathway (GA_4, GA_9 and GA_{34}) were present in stem internodes but not in young ears. Later members of the main GA pathway ($GA_{19} \rightarrow GA_{20} \rightarrow GA_1 \rightarrow GA_8$) were quantified by GC-selected ion monitoring in stem internodes and ears. In the *rht* (tall) line, $GA_{19} \ggg GA_{20} \approx GA_1 \ll GA_8$ during the main phase of expansion growth of both stem internodes and ears, suggesting that the conversion of GA_{19} to GA_{20} is a rate-limiting step on the GA pathway. In vegetative stem internodes, GA_1 concentration increased in proportion to the severity of the *Rht1* (semi-dwarf) and *Rht3* (dwarf) phenotypes but, at certain stages of *Rht3* internode development, the expected decrease in GA_{19} content, relative to the GA-responsive, *rht* (tall) line, did not occur and both GA_{19} and GA_1 accumulated. In very young ears of both tall and dwarf lines, the concentrations of GA_{20} and GA_8 were high, suggesting low 3β-(GA_{20} to GA_1) and high 2β-(GA_1 to GA_8) hydroxylase activities prior to the period of rachis elongation. The *Rht* alleles had no effect on either GA content or rates of ear rachis elongation during the main phase of ear growth, providing indirect evidence that *Rht* genes are not expressed in expanding wheat ears.

INTRODUCTION

Reduced height (*Rht*) semi-dwarfing genes have been incorporated into most high-yielding modern wheat varieties that formed the basis of the so-called "green revolution" [1]. Wild type, *rht1* = *rht3* (tall), *Rht1* (semi-dwarf) and *Rht3* (dwarf) genes are alternative alleles at the same locus on chromosome 4BS of wheat [1] and the degree of dwarfism conferred by these and certain other *Rht* alleles is associated with an increasing unresponsiveness of vegetative shoot tissues to the growth-promoting hormone, gibberellic acid (GA_3) [2]. The primary effect of these *Rht* alleles on shoot stature is to decrease the rate of expansion and final lengths of leaves and associated stem internodes [3, 4] by constraining wall extensibility of cells in the distal region of the expansion zone [5, 6]. Whereas the *Rht* gene product affects cells derived from the activity of intercalary meristems, there is less evidence that *Rht* genes are expressed in the shoot apical meristem.

The shoot apex of winter wheat undergoes a transition from the vegetative to the floral state following a period of low temperature (vernalisation) and increasing day-lengths. Outgrowth of foliar primordial ridges becomes arrested and the upper cauline ridges develop into the spikelets of the ear [7]. The presence of different *Rht* alleles has no effect on the rates of initiation or final numbers of leaves and spikelets produced in near-isogenic lines grown under natural conditions [8]. Since applied GAs can hasten, and inhibitors of GA biosynthesis delay, flower initiation in both tall and semi-dwarf wheats [7, 9], these observations suggest that, if changes in endogenous GAs or GA-responsiveness are part of the natural flower initiation process in wheat, the apical meristem of *Rht* genotypes is as sensitive to GA as *rht* (tall) lines. In addition, unlike the situation with vegetative stem internodes, the *Rht* alleles have no effect on the growth of the rachis internodes during the phase of rapid ear expansion, so that final ear lengths are similar in both *rht* and *Rht* genotypes [4].

Qualitative analyses of GAs in tissues of wheat seedlings have provided evidence for the identity of all members of the main early 13-hydroxylation GA

pathway ($GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20} \rightarrow GA_1 \rightarrow GA_8$) [3, 10–12]. Based on the relatively high abundance of GA_{19}, compared with GA_{20}, it was proposed that the conversion of GA_{19} to GA_{20} was a rate-limiting step on the pathway to bioactive GA_1 in seedlings grown under vernalisation conditions [10, 12]. As well as decreased shoot growth and GA-responsiveness, GA_1 has been shown to accumulate in vegetative tissues of several *Rht* genotypes [2, 13, 14]. In leaves of near-isogenic seedlings, the pool-sizes of GAs were $GA_{19} \gg GA_{20} = GA_1$ in *rht* (tall) lines, whereas $GA_{19} \approx GA_{20} \lll GA_1$ in the *Rht3* (dwarf) line [3]. These data supported the suggestion that the GA_{19} to GA_{20} step may be down-regulated in normal, GA-responsive lines as a consequence of GA action [3, 15].

The objectives of the present work were to identify GAs present in rapidly expanding stem internodes and young, developing ears of wheat, and to compare changes in GAs during vegetative internode and ear growth in lines containing different dwarfing alleles. In addition to examining the relationship between GA status and elongation growth of stem and rachis internodes, such an approach may lead to the identification of GAs with potential florigenic activity and may provide further evidence concerning the expression of *Rht* genes in developing wheat ears.

RESULTS AND DISCUSSION

Gibberellins were identified in young, developing ears at the start of the phase of rapid rachis elongation and in subtending vegetative stem internodes by full-scan GC-mass spectrometry as their methyl ester TMSi ether derivatives and comparison with library data [16]. Most GAs belonging to the early 13-hydroxylation GA pathway (GA_{53}, GA_{44}, GA_{19}, GA_{17}, GA_{20}, GA_{29}, GA_1, GA_8 and GA_3) were identified in rapidly expanding vegetative stem internodes (Table 1). The occurrence of 3-*epi* GA_1 in the presence of large amounts of GA_1 in these *Rht3* internodes may have been an artefact of work-up and GC analysis [17], since it was not detected during routine quantitative analysis using a different instrument. The identification of GA_9, GA_4 and GA_{34} in internodes suggests that the early non-hydroxylation GA pathway may also be operative in wheat shoots. These observations confirm and extend the range of GAs identified previously in wheat seedlings by full-scan GC-mass spectrometry [3, 11] and GC-selected ion monitoring [10–12]. We were unable to confirm the identification of GA_7 in wheat [11] and could find no evidence for the occurrence of GA_5, a potential biosynthetic intermediate between GA_{20} and GA_3, as already established in maize [18] and rice [19].

GAs belonging to the early 13-hydroxylation GA pathway were also identified in young, developing ears, although, in some cases, low abundance and the presence of interfering ions gave a poorer library fit than with the internode samples (Table 1). For example, GA_3 co-chromatographed with GA_{97} on a non-polar stationary phase but was resolved on a semi-polar one [15], although only the ions at *m/z* 504, 475, 387, 370 and 207 were of the expected intensities, compared with the standard. The identification of GA_{53} and GA_{44} in developing ears was strengthened by the occurrence of the corresponding 2β-hydroxylated compounds, GA_{97} and GA_{98} [20]. Interestingly, there was no evidence of members of the non-13-hydroxylation GA pathway at this stage of ear development, although they may occur later during anther formation, as in rice [21]. No novel GAs that may have been associated with spikelet and floret development were detected in young wheat ears. However, the growth promoters, GA_1 and GA_3, are also known to induce flowering in wheat [7, 9].

Initial quantification of GAs of the later stages of the main early 13-hydroxylation GA pathway ($GA_{19} \rightarrow GA_{20} \rightarrow GA_1 \rightarrow GA_8$) included a sample at a similar developmental stage as that used for qualitative analyses (39 days before anthesis). Because of the small size of young ears, material from the two tall lines was combined, as was that from the semi-dwarf and dwarf lines, whereas the adjacent upper expanding internodes from individual genotypes were examined. Relatively high concentrations of GA_{19} and GA_8, compared with GA_{20} and GA_1, were present in upper internodes of the two tall lines (Table 2), as observed previously in the expansion zone of leaves of the same genotype [3]. The presence of the *Rht1* (semi-dwarf) and *Rht3* (dwarf) alleles resulted in 4-fold and ca 20-fold increases, respectively, in GA_1 concentration, compared with the tall lines. However, the *Rht3* allele did not cause such a marked decrease in GA_{19} content, relative to *rht* (tall) lines, as observed previously in leaves [3], suggesting a change in regulation at an earlier step in the GA biosynthetic pathway.

The concentrations of GA_{20} and GA_8 were much higher in very young ears, harvested 46 days before anthesis (Table 3) than in subtending internodes (Table 2), whereas GA_{19} and GA_1 concentrations were similar in both tissues. Although insufficient internal standard was added to permit reliable quantification of GA_8 at the earliest stage of ear development, the results suggested high 2β-hydroxylase (GA_1 to GA_8) and low 3β-hydroxylase (GA_{20} to GA_1) activities in very young ears. One week later, 3β-hydroxylase activity appeared to increase (GA_{19}:GA_{20} ratio higher) and 2β-hydroxylase activity remained high. In marked contrast to the adjacent stem internode (Table 2), mechanisms maintaining relatively low concentrations of bioactive GA_1 appear to be operating in young developing ears of all three genotypes (Table 3). The extent of vascular connections and transport pathways between these organs at this stage of development are largely unknown.

In a more detailed developmental analysis, GAs were measured in the P-1 internode (one below the peduncle) and the rachis of the ear, since both tissues were undergoing expansion growth at a similar time

Table 1. Comparison of mass spectra of methyl ester TMSi ether derivatives of authentic gibberellins with those isolated from expanding stem internodes and young ears of *Rht3* wheat plants

Compound	KRI*		Relative intensities (% base peak) of characteristic ions *m/z*						
GA_1		Ions 506 $[M]^+$	491	448	447	377	376	313	207
Standard	2668	100	9	18	10	12	14	9	2
Internode	2676	100	18	19	10	14	17	12	38
Ear	2674	100	11	20	10	15	14	10	7
3-epi GA_1		Ions 506 $[M]^+$	491	459	448	431	376	313	208
Standard	2788	100	6	7	31	8	28	7	20
Internode	2796	100	9	9	31	8	29	9	15
GA_3		Ions 504 $[M]^-$	489	475	445	370	311	238	208
Standard	2692	100	7	12	12	24	14	21	37
Internode	2699	100	9	10	13	18	13	24	54
Ear	2678†	100	—	10	—	16	—	—	—
GA_4		Ions 418 $[M]^-$	386	328	289	284	261	233	225
Standard	2503	22	27	25	58	100	28	41	77
Internode	2515	18	19	22	59	100	33	42	86
GA_8		Ions 594 $[M]^+$	579	535	448	379	268	238	207
Standard	2818	100	5	6	14	7	5	13	18
Internode	2822	100	6	5	18	12	8	15	20
Ear	2821	100	8	7	18	11	7	17	26
GA_9‡		Ions 330 $[M]^+$	298	286	270	243	227	226	217
Standard	2203	6	100	18	99	52	59	61	32
Internode	2321	6	100	10	88	64	65	6	28
GA_{17}		Ions 492 $[M]^+$	460	433	373	293	251	208	207
Standard	2575	43	23	26	23	14	24	100	96
Internode	2582	26	23	23	23	8	18	100	81
Ear	2583	20	18	21	21	11	20	100	92
GA_{19}		Ions 462 $[M]^+$	434	402	374	345	285	239	208
Standard	2596	7	100	37	64	24	24	33	32
Internode	2607	5	100	36	61	27	24	24	45
Ear	2607	3	100	30	66	32	40	—	49
GA_{20}		Ions 418 $[M]^+$	403	375	359	301	235	207	
Standard	2482	100	15	46	12	12	8	30	
Internode	2494	100	15	60	14	16	9	31	
Ear	2493	100	15	55	18	13	15	46	
GA_{29}		Ions 506 $[M]^-$	491	447	389	375	303	235	207
Standard	2684	100	11	6	8	15	20	10	39
Internode	2689	100	—	8	5	6	23	17	100
Ear	2689	100	10	10	16	28	37	—	53
GA_{34}		Ions 506 $[M]^+$	431	416	372	261	229	223	201
Standard	2665	100	5	9	8	14	23	30	22
Internode	2672	100	5	6	9	4	14	24	7
GA_{44}		Ions 432 $[M]^+$	417	373	238	219	207		
Standard	2786	46	6	14	33	6	100		
Internode	2805	45	8	16	37	5	100		
Ear	2805	21	1	9	20	6	100		
GA_{53}		Ions 448 $[M]^+$	416	389	251	241	235	208	207
Standard	2497	63	19	32	24	34	19	89	100
Internode	2504	49	15	28	14	30	11	88	100
Ear	2505	100	28	72	56	89	—	43	—
GA_{97}		Ions 536 $[M]^+$	521	504	477	387	327	239	207
Standard	2695	26	5	8	9	7	8	41	100
Ear	2699	21	5	9	8	9	10	46	100
GA_{98}		Ions 520 $[M]^+$	505	433	343	299	238	208	207
Standard	2960	20	3	5	6	6	18	3	100
Ear	2966	71	13	14	18	18	49	73	100

* Kovats retention index. Standards analysed using OV1 and plant samples using BP1 stationary phases.

† Resolved from GA_{97} on BP5 GC stationary phase.

‡ As methyl ester.

Table 2. Gibberellin concentration (ng^{-1} g fr wt) in uppermost expanding stem internodes of wheat (cv. Maris Huntsman) containing *rht1*, *rht3* (tall), *Rht1* (semi-dwarf) and *Rht3* (dwarf) alleles

Time to anthesis (d)	Genotype	Mean shoot height (mm) (±S.E.)	GA_{19}	GA_{20}	GA_1	GA_8
46	*rht1*	70.2 (±8.2)	11.0	0.9	0.7	10.1
	rht3	61.5 (±7.3)	12.8	0.7	0.7	12.8
	Rht1	50.3 (±8.1)	11.0	1.8	3.1	12.4
	Rht3	29.0 (±6.2)	9.6	2.7	10.7	21.7
39	*rht1*	145.9 (±18.5)	10.1	0.9	0.5	8.1
	rht3	148.4 (±20.9)	11.0	1.0	0.5	9.1
	Rht1	119.5 (±16.0)	7.3	0.7	1.8	9.9
	Rht3	63.1 (±11.1)	6.3	3.1	12.7	8.5

Table 3. Gibberellin concentration (ng^{-1} g fr wt) in young developing ears of wheat (cv. Maris Huntsman) containing *rht1*, *rht3* (tall), *Rht1* (semi-dwarf) and *Rht3* (dwarf) alleles

Time to anthesis (d)	Genotype	Mean ear length (mm) (±S.E.)	GA_{19}	GA_{20}	GA_1	GA_8
46	*rht1*/*rht3*	4.3 (±0.8)	10.1	10.1	1.0	>55*
	Rht1/*Rht3*	4.7 (±1.1)	7.5	5.1	1.3	>45
39	*rht1*/*rht3*	13.5 (±2.7)	5.1	1.7	0.1	17.7
	Rht1/*Rht3*	13.1 (±1.8)	3.5	0.6	0.3	21.9

* Beyond upper range of calibration curve.

(Fig. 1). The *Rht* alleles decreased the rate of expansion of P-1 internodes, causing reductions in final lengths of 15% and 55% for *Rht1* and *Rht3* alleles, respectively, compared with *rht* (tall) genotypes. In contrast, the *Rht* alleles had no effect on the pattern of rachis expansion or final lengths of ears (Fig. 1), as observed previously [4]. The most notable feature of the GA analyses was that GA_1 accumulated *Rht3* >>> *Rht1* > *rht* in P-1 internodes during the linear phase of expansion, whereas GA_1 concentration remained low, and was similar, in ears of all three genotypes during the period of rachis elongation (Fig. 2), providing further indirect evidence that *Rht* genes are not expressed in developing ears. No significant genotypic differences in GAs were detected in ears, whereas the *Rht3* and, to a much lesser extent, the *Rht1* alleles affected the GA profile in P-1 internodes, compared with the *rht* (tall) genotype (Fig. 2). The accumulation of GA_1 was directly proportional to the severity of the *Rht* allele or the rate of expansion of the P-1 internode (Fig. 1). Previously, it was argued that the accumulation of GA_1 in *Rht3* wheat leaves was due to the release of the block on the GA_{19} to GA_{20} step that normally occurs as a consequence of GA action in GA-responsive, *rht* (tall) lines [3]. However, the situation in internodes appears to be more complex. Although GA_{19} concentrations were *rht* > *Rht1* >> *Rht3* in upper internodes at 39 days before anthesis (Table 2) and in P-1 internodes at the beginning of the phase of rapid expansion (Fig. 2), genotypic differences were not apparent at later stages of P-1 internode development.

Since genotypic differences in GA content may have been masked by the increasing proportion of mature tissue, GA distribution was examined in P-1 internodes at ca 25% final lengths. The concentration of GAs was much higher in the basal segments of both genotypes containing the intercalary meristem and expansion zone than in the more distal segment containing maturing tissues (Table 4). As expected, GA_1 accumulated in the *Rht3* (dwarf), compared with the *rht3* (tall), isoline but there was no decrease in the pool-size of GA_{19} in *Rht3* (dwarf) segments (Table 4), again confirming that factors in addition to GA-responsiveness may perturb the GA pathway, such that both GA_{19} and GA_1 accumulate in *Rht3* internodes at certain stages of development. It remains a paradox that GA_{19} accumulates in GA-responsive, *rht* (tall) wheat, since an expressed wheat GA 20-oxidase cDNA clone converts GA_{53} through to GA_{20} very efficiently (Appleford, Phillips, Hedden and Lenton, unpublished results).

Although the present experiments were not designed to test whether vegetative stem internodes of *rht* (tall) wheat were responsive to the presence of low concentrations of GA_1 (Tables 2 and 4; Fig. 2), indirect evidence suggesting that this was the case came from treatment of plants with a single dose of the early-step GA-biosynthesis inhibitor, paclobutrazol, at the beginning of the phase of rapid stem

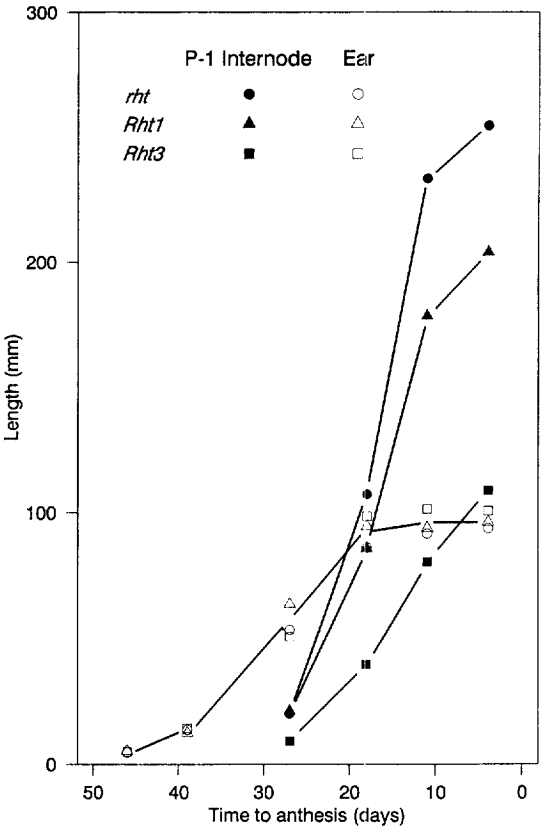

Fig. 1. Growth of P-1 internodes (closed symbols) and ears (open symbols) of winter wheat (cv. Maris Huntsman) containing *rht* (circles), *Rht1* (triangles) and *Rht3* (squares) alleles.

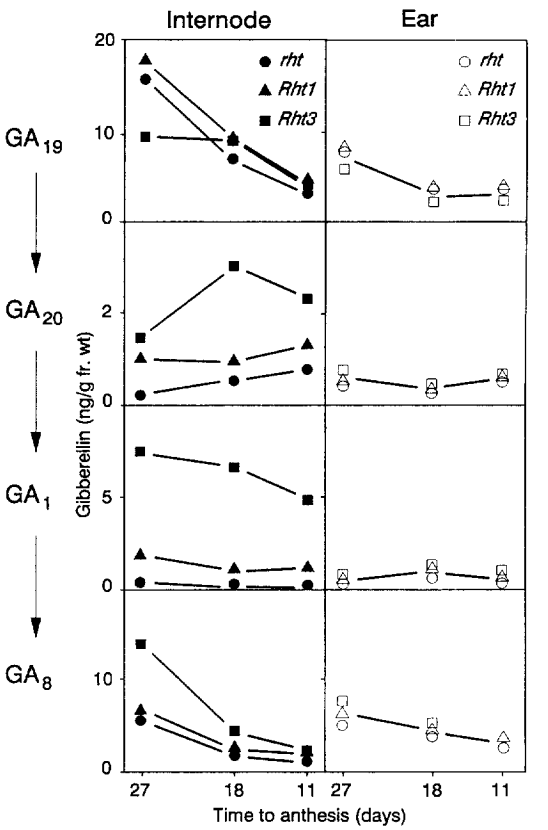

Fig. 2. Changes in GA concentrations in P-1 internodes (closed symbols) and ears (open symbols) of winter wheat (cv. Maris Huntsman) containing *rht* (circles), *Rht1* (triangles) and *Rht3* (squares) alleles.

extension, six weeks before anthesis. Final lengths of P-2, P-1 internodes and peduncle were decreased by 65%, 49% and 16%, respectively, compared with untreated controls, but there was no effect on final ear length (Webb, Appleford and Lenton, unpublished results). It is suggested that the rachis of the ear may not contain GA-responsive cell types that are characteristic products of the intercalary meristem in vegetative stem internodes.

Table 4. Gibberellin concentration ($ng^{-1}g$ fr wt) in rapidly expanding P-1 internodes of wheat (cv. Maris Huntsman) containing *rht3* (tall) and *Rht3* (dwarf) alleles

Genotype	Segment length (mm)	GA_{19}	GA_{20}	GA_1	GA_8
rht3	0–24	12.1	0.3	0.5	3.2
Rht3	0–11	17.4	0.9	18.7	6.3
rht3	24–48	4.3	0.2	0.1	1.0
Rht3	11–22	5.5	0.6	5.3	2.5

EXPERIMENTAL

Plant material

Near-isogenic pairs of lines, homozygous for either *rht1* (tall) or *Rht1* (semi-dwarf) and *rht3* (tall) or *Rht3* (dwarf) alleles, in the background winter wheat (*Triticum aestivum* L. cv. Maris Huntsman) were generously supplied by Prof. M. D. Gale, John Innes Centre for Plant Science Research, Norwich [1]. Seeds were sown in pots (27 cm diam.) containing a loam-based compost (10 l) with Osmocote slow-release fertiliser granules. Pots were transferred outside to a plunge bed and plants were thinned to 15 per pot at the seedling stage. At various times throughout the growing season, plants were harvested, dissected into the required tissues for subsequent GA analysis, plunged into liquid N_2, weighed and stored in MeOH at $-25°$. Mean lengths ($\pm$S.E.), fr. and dry wts were determined on a subset of stems, individual internodes and ears.

Identification of GAs in stem internodes and ears

Upper expanding *Rht3* stem internodes (5–20 mm, 25.3 g) and adjacent developing ears (10–20 mm, 8.1 g) were homogenised and small amounts (ca 800 Bq

each) of [1,2-$^3H_2$]GA_1, [1,2,3-$^3H_3$]GA_{20}, [1,2-$^3H_2$]GA_4 and [2,3-$^3H_2$]GA_9 were added and stirred overnight at 4° [3]. After filtration, the residues were re-extracted for 3 h at room temp. The combined MeOH extracts were evapd under red. pres. at <35° and the aq. residue adjusted to pH 3 (2 M HCl) and partitioned against EtOAc (4 × equal vols). The combined EtOAc fr. was partitioned against 5% w/v $NaHCO_3$ (3 × 0.2 vol) and the EtOAc-solubles re-extracted from the acidified aq. phase and evapd to dryness (internodes 20.1 mg, ears 2.6 mg). After purification by QAE-Sephadex anion-exchange, C_{18} Sep-Pak cartridge chromatography and reverse-phase HPLC [3, 22], grouped HPLC frs were methylated (CH_2N_2) dried and partitioned between H_2O (500 μl) and EtOAc (3 × 1 vol). The organic phases were passed through an NH_2 Bond Elut (100 mg) column, evapd to dryness, trimethylsilylated and analysed by GC-MS [22], but using a BP-1 (SGE) capillary column (0.1 μM film thickness). Differences in Kovats retention indices between standards in the ref. library [16] and the wheat GAs (Table 1) can be accounted for by differences in column phase and film thickness.

Quantification of GAs in upper internodes and young ears

Samples consisted of 100 upper stem internodes (3.1–4.2 g) and ears (0.34–0.45 g) per genotype at 46 days before anthesis and 70 internodes (4.3–6.9 g) and ears (1.3–1.4 g) per genotype at 39 days before anthesis. In addition to tritiated GAs, [17-$^2H_2$]GA_{19}, [17-$^2H_2$]GA_{20}, [17-$^2H_2$]GA_1 (from Prof. L. Mander, Australian National University of Canberra) and [17-^{13}C]GA_8 (gift from Prof. B. Phinney, University of California) were added as int. standards. The amounts added depended on genotype, tissue and sample wt. For internodes, amounts added were based on preliminary analyses of lower internodes (data not shown) and were within the recommended analyte : int. standard ratio, 0.1–4.0 of the calibration curves [23]. For ears, the data presented in Table 3 were based on a single analysis and were beyond the upper range of the calibration curve for GA_8 in the sample harvested 46 days before anthesis. Following homogenisation and purification, as described above, samples were analysed by GC-SIM [3].

Quantification of GAs in P-1 internodes and expanding ears

At 27 days before anthesis, samples of 50 P-1 internodes (4.7–10.3 g) and ears (13.0–14.2 g) per genotype were harvested, whereas the corresponding samples at 18 days before anthesis consisted of 50 P-1 internodes (22.4–52.3 g) and ears (48.0–56.1 g) per genotype. The samples at 11 days before anthesis contained 30 P-1 internodes (8.8–26.5 g) and 10 ears (18.3–20.4 g) per genotype. P-1 internodes harvested at ca 25% final length (*rht3* 55–75 mm, *Rht3* 25–35 mm) were subdivided into lower (*rht3* 0–24 mm, *Rht3* 0–11 mm), containing intervalary meristem and expansion zone (*rht3* 32.1 g, *Rht3* 9.9 g) and equivalent upper segments containing more mature tissue (*rht3* 25.7 g, *Rht3* 11.6 g). Following addition of int. standards, samples were worked-up and analysed, as described above. For clarity, means of the GA analysis of P-1 internodes and ears from the two independent tall lines are presented in Fig. 2, since replication was good, as observed previously with upper internodes (Table 2).

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