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Genetic Regulation of Development in *Sorghum bicolor*[1]

V. The ma_3^R Allele Results in Gibberellin Enrichment

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ABSTRACT

***Sorghum bicolor* genotypes, near isogenic with different alleles at the third maturity locus, were compared for development, for responsiveness to GA_3 and a GA synthesis inhibitor, and occurrence and concentrations of endogenous GAs, IAA, and ABA. At 14 days the genotype 58M ($ma_3^Rma_3^R$) exhibited 2.5-fold greater culm height, 1.75-fold greater total height, and 1.38-fold greater dry weight than 90M (ma_3ma_3) or 100M (Ma_3Ma_3). All three genotypes exhibited similar shoot elongation in response to GA_3, and 58M showed GA_3-mediated hastening of floral initiation when harvested at day 18 or 21. Both 90M and 100M had exhibited hastening of floral initiation by GA_3 previously, at later application dates. Tetcyclacis reduced height, promoted tillering, and delayed flowering of 58M resulting in plants which were near phenocopies of 90M and 100M. Based on bioassay activity, HPLC retention times, cochromatography with $^2H_2$-labeled standards on capillary column GC and matching mass spectrometer fragmentation patterns (ions [m/z] and relative abundances), GA_1, GA_{19}, GA_{20}, GA_{53}, and GA_3 were identified in extracts of all three genotypes. In addition, based on published Kovats retention index values and correspondence in ion masses and relative abundances, GA_{44} and GA_{17} were detected. Quantitation was based on recovery of coinjected, $^2H_2$-labeled standards. In 14 day-old-plants, total GA-like bioactivity and GA_1 concentrations (nanograms GA/gram dry weight) were two- to six-fold higher in 58M than 90M and 100M in leaf blades, apex samples, and whole plants while concentrations in culms were similar. Similar trends occurred if data were expressed on a per plant basis. GA_1 concentrations for whole plants were about two-fold higher in 58M than 90M and 100M from day 7 to day 14. Concentrations of ABA and IAA did not vary between the genotypes. The results indicate the mutant allele ma_3^R causes a two- to six-fold increase in GA_1 concentrations, does not result in a GA-receptor or transduction mutation and is associated with phenotypic characteristics that can be enhanced by GA_3 and reduced by GA synthesis inhibitor. These observations support the hypothesis that the allele ma_3^R causes an overproduction of GAs which results in altered leaf morphology, reduced tillering, earlier flowering, and other phenotypic differences between 58M and 90M or 100M.**

Sorghum bicolor (L.) Moench is a short day grass of tropical origin that is now grown agriculturally at latitudes far removed from the equator. This is possible because mutations have occurred that reduce the night length required for floral initiation (19). In the milo-group of sorghums three genes have been identified that regulate photoperiod sensitivity (19–21). The alleles at the three loci segregate simply and independently (19–21), indicating a classical genetic system. This system has the important advantage that rather than expressing photoperiodism as an all or none characteristic, eleven genotypes, which vary in alleles at three loci, produce quantitative variation in photoperiodism ranging from essentially nonphotoperiodic to very photoperiodic genotypes–those that require the longer nights of late summer or early fall to induce flowering (15, 20, 21).

All of the mutations of the maturity genes are nonlethal, but one, ma_3^R, is unique in that it has the most striking effect upon the photoperiod requirement and morphology (15, 21). Plants containing the ma_3^R allele are essentially nonphotoperiodic and seedlings exhibit dramatically increased plant height, leaf blade length, leaf sheath length, and reduced numbers of tillers and adventitious roots (15). Non-ma_3^R containing genotypes can be made to appear like ma_3^R containing genotypes by application of GA_3 (16), implying that the ma_3^R allele may alter GA levels, sensitivity, and/or metabolism. Furthermore, the characteristic morphology of ma_3^R genotypes and non-ma_3^R genotypes treated with GA_3 is typical of the response of many plants to GA_3 (see review in ref. 16). In maize and rice, dwarf mutants exhibit reduced leaf sheath and leaf blade lengths as a result of reduced GA levels (17), and elongation of grass leaf sheaths is a classical bioassay for GAs (13, 17). Thus, there is indirect evidence that the ma_3^R allele may cause elevated GA levels and this hypothesis was proposed earlier (16, 21).

We report data on the identity and amounts of gibberellins in three sorghum genotypes differing at maturity locus three. We also report that plants containing the ma_3^R allele are both GA_3 responsive and growth retardant responsive. The ma_3^R allele apparently has the rarely seen effect of elevating gibberellin levels in a plant which is not a receptor or response transduction mutant.

MATERIALS AND METHODS

Plant Material

Seeds of *Sorghum bicolor* L. (Moench) were initially supplied by J. R. Quinby, Texas Agricultural Experiment Station,

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Lubbock-Halfway Center, Plainview, TX. The milo genotypes employed in the present study are near isogenic except for the following differences at the third maturity gene locus: 100M, Ma_3Ma_3; 90M, ma_3ma_3; 58M, $ma_3^Rma_3^R$ (19–21). Seeds were germinated and grown in 20 cm diameter pots filled with a mix developed locally by K. F. Schertz to minimize chlorosis problems in pot-grown sorghum (18.9 L peat, 9.45 L vermiculite, 9.45 L perlite, plus 225 g Osmotcote [14–14–14 controlled released fertilizer; Sierra Chemical Co., Milpitas, CA], 115 g dolomite, 70 g superphosphate, and 195 g gypsum; Fe chelate and $ZnSO_4$ were not added but supplied with initial and weekly watering with Hoagland solution). Seedlings were watered as required with distilled water. Except as noted below, plants were grown in EGC controlled environment chambers with 12 h photoperiods, 30°C day/20°C night temperatures and 80% RH day/100% RH night. PPFD was 800 to 1000 μmol m^{-2} s^{-1} at the plant canopy level, measured with a Li-Cor model 185 photometer fitted with a model 190 quantum sensor. CO_2 levels in building air circulated through the chambers ranged from 700 ppm at the end of the day to 500 ppm during much of the night, measured with an ADC infrared gas analyzer. While the studies reported here were in progress, the building fresh air mix was changed and daytime peak CO_2 levels decreased to 580 ppm with nighttime levels falling to 350 ppm. In the experiment utilizing 7- and 10-d old plants, the seedlings were grown in a Conviron model GR48 growth chamber at Great Lakes Forestry Centre, Sault St. Marie, Ontario, Canada. Lighting was provided by a mixture of metal halide and quartz iodine lights giving a PPFD of 400 μmol m^{-2} s^{-1} at the soil level. Temperatures were maintained at 30°C day/20°C night with a 12 h photoperiod and the RH was 60% day/90% night.

GA_3 was applied by micrometer-activated syringe (L. S. Starrett Co., Athol, MA) in concentrations and volumes indicated in "Results." Tetcyclacis (BASF, Ludwigshafen, Federal Republic of Germany) was applied in the nutrient solution to plants in water culture at concentrations shown in "Results."

After emergence and thinning, plants were measured from the soil level to the tip of the tallest leaf (total height) and to the uppermost leaf collar (leaf sheath or culm length). Days to floral initiation were determined by examining a longitudinal section through the apical meristem under a dissecting microscope as previously described (15, 29). At harvest the plants were separated into leaf blades, culms, and the basal portion of the shoot containing the apical meristem; older senescing leaves were discarded. The plant tissue was frozen at −80°C and lyophilized.

Extraction and Purification of Endogenous GAs

Lyophilized tissue samples were weighed and ground to a fine powder in a chilled mortar and pestle with the aid of acid-washed sand. Alternatively, if the total sample weight exceeded the capacity of the extraction procedure, the total sample was ground to a fine powder in a blender and a 1 g subsample was processed as described below. The powdered tissue was extracted with 10 to 20 mL of cold 80% aqueous CH_3OH and approximately 50,000 dpm each of [1,2-^{3}H]GA_1 (31.5 Ci $mmol^{-1}$, Amersham) and [1,2-^{3}H]GA_4 (31.5 Ci $mmol^{-1}$, Amersham) were added as internal standards. The tissue residue was filtered off and then reextracted overnight with agitation at 5°C with 15 to 25 mL of the extraction solvent. The methanolic extracts were combined and adjusted to pH 6.5 to 7.0 with 2 N NH_4OH.

The extracts were purified by a procedure modified from Koshioka *et al.* (8). Briefly, the 80% CH_3OH extracts were diluted with water to equal 60% CH_3OH, and passed through a 10 g column of Davisil C_{18} (90–130 μm, 60 Å pore size, Alltech) and then rinsed two times with 20 mL of 60% CH_3OH. GA precursors (kaurene and kaurenoic acid) were eluted with 50 mL of 85% CH_3OH. The 60% CH_3OH and 85% CH_3OH column eluates were reduced to the aqueous phase on a rotary flash evaporator, frozen, and lyophilized. The residue from the 60% eluate was solubilized in a small amount of water (approximately 1 mL) and 50 mL of CH_3OH added, followed by 50 mL of EtOAc.[3] The precipitate formed after the addition of the EtOAc was removed by vacuum filtration through a 0.2 μm Nylon 66 membrane filter (MSI, Fisher Scientific) and the precipitate washed with additional 1:1 (v:v) EtOAc:CH_3OH. The filtrate was then taken to dryness on the rotary evaporator. The resulting residue was solubilized in a small volume of 80% CH_3OH and dried onto 1 g of celite with a warm air stream and gentle warming from a hot plate. This celite was then loaded onto a 5 g SiO_2 partition column, and the free GAs (except for highly polyhydroxylated GAs, for example, GA_{32}) were eluted with 70 mL of 95:5 formate-saturated EtOAc:hexane. GA glucosyl conjugates were extracted from the SiO_2 column by slurrying the column packing with 100 mL CH_3OH and filtering off the silica particles.

HPLC and GA Bioassay

The three fractions resulting from the above procedure, precursors, free acid GAs, and GA glucosyl conjugates (with highly polyhydroxylated GAs), were dried on a flash evaporator and prepared for reverse-phase C_{18} HPLC (9). The HPLC system consisted of Waters model 680 Automated Gradient Controller, U6K injector, model 6000A pump, model 45 pump, and an Isco fraction collector. The free acid and glucosyl conjugate fractions were chromatographed on a 3.9 × 300 mm μ Bondapak C_{18} column (Waters) eluted at 1.5 mL min^{-1} with the following gradient: 0 to 5 min, isocratic 28% CH_3OH in 1% aqueous acetic acid; 5 to 35 min, linear gradient from 28 to 86% CH_3OH; 35 to 36 min, 86 to 100% CH_3OH; 36 to 40 min, isocratic 100% CH_3OH. Precursor fractions were chromatographed with the following gradient: 0 to 15 min, isocratic 46% CH_3OH in 1% aqueous acetic acid; 15 to 30 min, linear gradient from 45% CH_3OH to 100% CH_3OH. Fractions were collected every 2 min. The fractions were dried and GA-like biological activity detected with the dwarf rice bioassay (13) in serial dilution, modified in that first leaf sheath length was measured after 48 h. The GA glucosyl conjugate fractions were assayed with the dwarf

[3] Abbreviations: EtOAc, ethylacetate; BHT, butylated hydroxytoluene; BSTFA, *Bis*(trimethylsilyl)-trifluoroacetamide; GC-MS-SIM, gas chromatography-mass spectrometry-selected ion monitoring; TMCS, trimethyl chlorosilane; KRI, Kovats retention index.

rice immersion assay (14). To further purify and separate cochromatographing GAs, fractions exhibiting biological activity were grouped within samples and rechromatographed on a 4.6 × 150 mm 10 μm Nucleosil $N(CH_3)_2$ column (Alltech) eluted isocratically with 0.1% acetic acid in CH_3OH (30). Fractions were collected every 2 min and rebioassayed. Recovery of the [^{3}H] GAs used as internal standards was determined after HPLC by liquid scintillation spectrometry from aliquots of the fractions.

In the experiment with 7- and 10-d old plants, the free acid GAs were chromatographed on a Waters (Milford, MA) HPLC system composed of a model 820FC data station, 2 model 501 pumps, a model U6K injector, and an Isco fraction collector. The column was a μBondapak C_{18} radial compression cartridge (8 × 100 mm) in a RCM 8 × 10 radial compression module. The column was eluted at 3 mL min^{-1} with the following gradient: 0 to 5 min, 20% CH_3OH in 1% aqueous acetic acid; 5 to 30 min, linear gradient from 20 to 84% CH_3OH; 30 to 32 min, linear gradient from 84 to 100% CH_3OH; 32 to 40 min, 100% CH_3OH. After determination of biologically active fractions with the dwarf rice assay, appropriate fraction groupings were rechromatographed on a 4.6 × 150 mm 10 μm Nucleosil $N(CH_3)_2$ column isocratically eluted at 1 mL min^{-1} with 0.1% acetic acid in CH_3OH and biological activity determined with the dwarf rice assay (13).

GC-MS-SIM

Fractions exhibiting significant biological activity or those corresponding to the expected retention times of biologically inactive GAs (*e.g.* GA_8) were pooled, taken to dryness, and transferred to 1 mL conical vials. To accurately determine GA amounts, 50 ng of an appropriate deuterated GA ([17, 17-$^2H_2$]$GA_{1,19,20,53}$) was added to the vial before derivatization. The methyl esters were prepared with ethereal diazomethane and, after being dried with N_2, freeze-dried, and resolubilized in 50 μL pyridine, the samples were silylated with 100 μL of BSTFA with 1% TMCS (Pierce Chemical Co.). The samples were then reduced to dryness with N_2 and solubilized in dry CH_2Cl_2. Approximately one-third to one-fifth of each sample was injected on-column to a 12 m, 0.2 mm i.d., 0.33 μm film thickness methyl silicone fused silica capillary column (HP-1, Hewlett-Packard). Hydrocarbon standards, prepared from 'Parafilm', were coinjected with the sample to determine KRI. The GC (model 5890, Hewlett-Packard) oven was programmed for a 1 min hold at 60°C, then to rise at 15°C min^{-1} to 200°C followed by 5°C min^{-1} to 270°C. Helium head pressure was adjusted to give a linear velocity through the column of 40 cm s^{-1}. The GC was directly interfaced to a 5970B Mass Selective Detector (Hewlett-Packard). The MS operating parameters were: interface and source temperature, 280°C; 70 eV ionizing voltage; dwell time, 100 ms. The instrument was regularly tuned with the Autotune program and operated at a photomultiplier voltage 200 mV above the recommended voltage.

Quantitation and Characterization of Endogenous GAs

In each GC-MS-SIM analysis, three ions of the added [^{2}H] GA internal standard and three ions of the endogenous GA were measured simulatenously. Because nonlabeled GA standards were not available and standard curves could not be constructed (5), quantitation was based on the peak area ratio of deuterated to nondeuterated GA after correcting the nondeuterated peak area for any contribution from the deuterated species determined from standard runs. Contribution of native GAs to the deuterated species was minor because of the amount of deuterated standard added (50 ng) and this correction was not done. Usually the most prominent ion measured (usually M^+) was used for quantitation and the other ions and KRI for identity confirmation. Values obtained from the GC-MS-SIM analysis were further corrected for sample recovery based on the recovery of [^{3}H]GA_1 (for GA_1 and GA_3) and [^{3}H]GA_4 (GA_{20}, GA_{19}, GA_{53}), determined prior to derivitization. Recoveries of [^{3}H]GA_1 averaged 27.4%, with a range of 21.5 to 33.4%. [^{3}H]GA_4 recoveries averaged 32.7%, with a range of 20.8 to 40.2%. Thus, the values presented herein are not absolute but reasonably accurate relative values.

Confidence in the GA data is based on the extraction of multiple samples from many different experiments, the inclusion of ^{3}H-labeled internal standards allowing us to use bioassay as a highly sensitive qualitative tool, the close correspondence between the bioassay and GC-MS-SIM results, and that a time course of GA levels *versus* age consistently showed a higher level of GA_1 in the tall genotype. While repli cated experiments were not performed for most time points, 12-d-old plants were assayed two additional times with similar trends in GA_1 levels, but other samples from these experiments were lost and hence the data have not been presented. Preliminary experiments where the analysis was not carried past bioassay consistently showed greater GA_1-like activity in extracts of 58M.

Extraction, Purification, and Quantitation of ABA and IAA

Analysis of ABA and IAA was adapted from published methods (2, 24). Freeze-dried tissue samples (250 mg subsamples of leaves and culms, total sample of apices) were ground in a mortar and pestle. The powdered residue was extracted with 10 mL of 80:20 (v:v) CH_3OH:0.01 M ammonium acetate pH 6.5 containing 10 μM BHT. Approximately 50,000 dpm each of DL-*cis,trans*[G-^{3}H] ABA (33.2 Ci mmol^{-1}, Amersham) and [5-(*n*) -^{3}H] IAA (34.9 Ci mmol^{-1}, Research Products International Corp.) were added to the tissue slurry for chromatographic markers and 100 ng each of [$^2H_6$] ABA and [$^{13}C_6$] IAA for quantitation by GC-MS-SIM. The tissue residue was filtered off and reextracted overnight at 5°C on a shaker. The methanolic extracts were combined and reduced to the aqueous phase on a rotary evaporator at 35°C after which the aqueous phase was centrifuged at 10,000*g* for 30 min. The supernatant was decanted, vacuum-filtered through a 0.2 μm nylon 66 membrane filter (MSI, Fisher Scientific), and then passed through a 3 mL bed volume DEAE-Sephadex (A-25, Sigma) column, preswollen and equilibrated to 0.01 M ammonium acetate, followed by a 5 mL rinse of 0.01 M ammonium acetate. The ABA and IAA were eluted from the DEAE column onto a C_{18} Sep-Pak cartridge (Waters) with 25 mL of 1 M acetic acid. The Sep-Pak was detached and washed with 5 mL of water, and the ABA and IAA eluted with 5 mL of CH_3OH. The CH_3OH was removed with a N_2 stream, and

the sample was solubilized in 10% CH_3OH with 0.1% acetic acid, filtered, and injected onto the HPLC. The extract was chromatographed on a 3 μm Zorbax C_8 column (6.2 × 80 mm, DuPont) with the following program: 0 to 5 min, isocratic 10% CH_3OH in 0.1% acetic acid; 5 to 15 min, 10 to 37% CH_3OH; 15 to 30 min, 37 to 46% CH_3OH; 30 to 35 min, 46 to 100% CH_3OH. Fractions (1 min) corresponding to the radioactive markers (fractions 16, 17 for ABA and fractions 22, 23 for IAA) were combined and taken to dryness with a rotary test tube evaporator (Buchler Inst.), and the residue was transferred to reaction vials, solubilized in 25 μL of CH_3OH, and methylated with ethereal diazomethane. The samples were dried with N_2, solubilized in dry cyclohexane, and then injected (1–2 μL) on-column in the GC-MS instrument described above with the following program: after a 1 min hold at 80°C, 20°C min^{-1} to 250°C. Under these conditions IAA eluted at 7.1 min and ABA at 9.3 min. Endogenous IAA was quantitated by monitoring three ions of the internal standard and three for the endogenous compound [195,136,109([$^{13}C_6$]IAA); 189,130,103(IAA)] and the resulting peak area ratios were compared to a standard curve to calculate endogenous IAA (2). ABA was quantitated in a similar manner except for the ions monitored (194,166,138,[$^2H_6$] ABA; 190,162,134,ABA) and the lack of sufficient [$^2H_6$] ABA precluded the construction of a standard curve so that quantitation was based on peak area ratios after correcting for the contribution of ions from one species to another.

RESULTS

Plants for these experiments were grown under higher light intensities, lower plant densities, and higher CO_2 levels than those for our previous study (15), which apparently allowed a more complete expression of the differences in genetic potential between the ma_3^R and non-ma_3^R genotypes. Culm height, which represents in seedlings the sum of the leaf sheath lengths, leaf blade length, and total height were greater in 58M than 90M and 100M (Fig. 1). Culm height and total height were equal for 90M and 100M but greater for 58M in the present study than in the previous one (*cf.* Fig. 1 here with Fig. 3 in ref. 15). Culm height of 58M was 2.5-fold greater than 100M and total height of 58M was 1.75-fold greater than 100M in the present study (Fig. 1). Previously, both comparisons were only 1.35-fold greater in 58M (15). In addition, the dry weight per plant of 58M at 14 d was 1.38-fold greater than that of 90M and 100M (see later), whereas previously dry weights were equal (15). Also, genotypes 90M and 100M had more tillers (Table I) and adventitious roots (data not given) per plant than 58M.

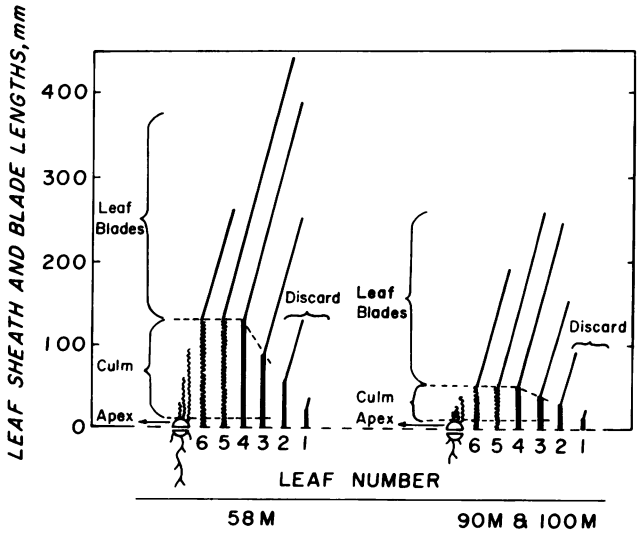

Figure 1. Schematic illustration of tissue included in samples designated apices, culms, and leaves. Leaf blade and culm length, except for leaves not yet exserted from the whorl, are drawn to scale for size at harvest on d 14. Data are from a typical experiment. In repeated tests culm and leaf height were approximately 120 and 400 mm for 58M and 50 and 250 mm for 90M and 100 M at d 14 and these relative differences persisted from d 6 to d 21 (data not given).

Table I. *Dry Weight of Sorghum Plants at 14 d after Planting*
Weights determined after freeze drying.

Measurement	Genotypes		
	58M	90M	100M
No. of plants	60	57	62
Dry wt (g)			
Leaves	8.71	6.49	7.47
Culms	4.30	2.01	2.23
Apices	0.52	0.57	0.64
Total	13.53	9.07	10.33
Average wt per plant (g)	0.23	0.16	0.17

As noted previously (15), rapid floral initiation in 58M began around d 18 to 20, and the population was 100% initiated by d 24 (Fig. 2). No 90M or 100M plant initiated near this time; the average date for 50% floral initiation for 90M and 100M in our growth rooms was 42 d if plants were shifted from 12 to 10 h photoperiods on d 35 (Fig. 2).

To verify that 58M is not a GA receptor or response mutant, the three genotypes were treated with GA_3 at levels which promoted shoot growth. GA_3 promoted elongation of leaf sheaths and internodes (total culm height) in all three genotypes (Table II). In both experiments GA_3 hastened floral initiation in 58M alone (Table III). Due to the early initiation in ma_3^R-containing genotypes, promotion of floral initiation with GA_3 had not been attempted earlier (16), but promotion in non-ma_3^R-containing genotypes with older plants had been demonstrated frequently (16, 29). In an additional test, plants were grown in solution culture and subjected to the GA-synthesis inhibitor tetcyclacis. At 31 d, 58M alone had undergone floral initiation, but increasing levels of tetcyclacis reduced culm height, total height, and floral initiation while promoting tillering (Table IV). Thus, presumed inhibition of GA biosynthesis made 58M more like the phenotype of 90M and 100M in leaf morphology, tillering, and floral initiation.

The only fraction from the extraction and purification procedure consistently exhibiting significant GA-like biological activity was the free GA fraction. The precursor and conjugate fractions were not analyzed further. The free GA extracts of 14-d-old plants showed three major peaks of GA-like activity in the dwarf rice bioassay (Fig. 3, panel 1). When

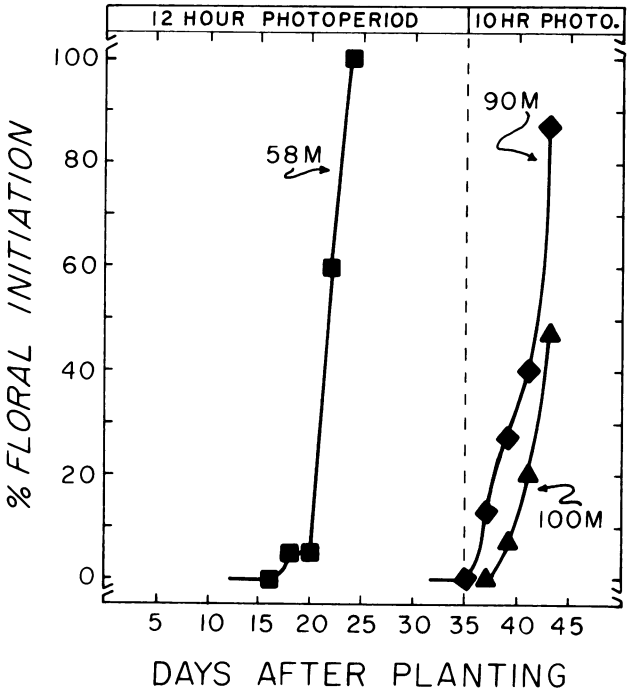

Figure 2. The floral initiation state of the apical meristem of 58M, 90M, and 100M plants in a controlled environment room under 12 h photoperiods. At d 35 the photoperiod was reduced to 10 h. Five plants were examined per day.

fractions comprising peaks B and C were rechromatographed on a Nucleosil $N(CH_3)_2$ column, each was resolved into several discrete peaks of GA-like activity (Fig. 3, panels 2 and 3). Fractions B3, C1, C2, C3, and D were derivatized and subjected to GC-MS-SIM. GA_1, GA_{19}, GA_{20}, and GA_{53} were identified from B3, C3, C2, and D, respectively, based on HPLC retention times, cochromatography on GC with ^2H-labeled standards, similar KRI values from the same MS, and correspondence in ion masses and relative abundances of three major ions for each GA with its deuterated standard

Table II. *Effect of GA_3 on Culm Height of Sorghum Plants*

In experiment 1, 3×10^{-3} M GA_3 in aqueous 10% ethanol was applied 50 μL/plant on d 10, 11, and 13, and 100 μL on d 14 and 17 (0.35 mg total GA_3 $plant^{-1}$). In experiment 2, 3×10^{-3} M GA_3 in aqueous 40% CH_3OH was applied 10 μL/plant on d 7 and 8, 20 μL on d 9, and 50 μL on d 14 and 16 (0.17 mg total GA_3 $plant^{-1}$).

Genotype	Treatment	Culm Height[a]		GA_3/Control
		Expt. 1[b]	Expt. 2[c]	
		mm		*Avg, %*
58M	GA_3	305	251	146
	Control	210	172	
90M	GA_3	197	160	156
	Control	134	99	
100M	GA_3	197	169	147
	Control	139	111	

[a] Measured to top of leaf sheath of youngest collared leaf. [b] Ended 19 d after planting. [c] Ended 18 d after planting.

Table III. *Effect of GA_3 Treatment on Floral Initiation and Floral Development in Sorghum Maturity Genotype 58M*

Treatment details and growth data are in Table II. There was no floral initiation of 90M or 100M in either experiment.

Treatment	Length of Inflorescence		Floral Stage (ref. 29)
	Expt. 1_a	Expt. 2_b	
	mm		
Control	1.3 ± 0.34	0.2	1.6
GA_3	2.4 ± 0.36	1.28	5.8

[a] Plants harvested on 21 d after planting, average of 10 plants examined for each genotype. [b] Plants harvested on 18 d after planting, average of 19 plants examined for each genotype.

(Table V). These data are from a single set of samples (apex tissues, 14-d-old plants), and similar data were obtained from samples of other tissues and all genotypes. However, within each set of samples a few would yield a more perfect match between ion masses and relative abundances for standards and endogenous GAs. Thus, the identification of endogenous GAs was repeated in each set of samples, but occassionally, for individual compounds, identification was more perfect than in the single set illustrated (Table V).

Concurrently with these experiments, GA analyses were conducted in the same laboratory with soybean tissue and GA_3 was not detected (1), leading us to believe that the ion m/z 504 in the sorghum samples did not come from GA_3 contamination of glassware, solvents, or related components of the purification procedure. Subsequently, we obtained a $^2H_2$-labeled GA_3 standard and found that an endogenous compound coelutes on capillary GC with the standard and produces three ions with Table VI matching m/z and relative abundances (Table VI). Thus, GA_3 is also present in sorghum; on HPLC GA_3 migrates with GA_1 and occurs in the fraction labeled B3 (Fig. 3). Unfortunately, the quantitative estimates of GA_3 activity given in this paper are based on compari son with the $[^2H_2]GA_1$ internal standard employed before the $^2H_2$-GA_3 standard was available. For that reason they must be viewed as preliminary.

Authentic standards for GA_{44} and GA_{17} were not available, but fraction C1 (Fig. 3, panel 3) contained ions m/z 432, 417, 373 typical of GA_{44} and ions m/z 492, 460, and 432 typical of GA_{17} (Table V). Based on fragmentation patterns and KRIs for GA_{44} and GA_{17} in the literature (5), we tentatively identify them as occurring in fraction C1.

Semiquantitative analysis of endogenous GAs, based on the $^2H_2$-labeled standard for each GA, was done for apex, culms, and leaf blades from plants harvested on d 14. Both total bioassay activity and GC-MS estimations indicated that concentrations of GAs were lower in leaves than culms or apex (Tables VII and VIII). The concentrations of total GA-like bioactivity (Table VII) and GA_1 (Table VIII) were always from two- to sixfold higher in 58M than the average of 90M and 100M, except in culms, where concentrations were similar but the weight of tissue and thus total GA amounts were higher in 58M. When the GA concentration data are calculated on an amount per plant basis, 58M contained more GA_1 by about twofold for plant totals (Table VIII).

Table IV. *Effect of Tetcyclacis in the Nutrient Solution on Growth and Development of Sorghum Maturity Genotypes after 31 d (average for five plants per treatment)*

Treatment	Genotype	Culm Height	Total Height	Number Tillers/Plant	Floral Initiation
		mm			%
Control	58M	154	616	0	100
	90M	102	566	4	0
	100M	108	544	4	0
Tetcyclacis					
5×10^{-9} M	58M	148	582	0	100
5×10^{-8} M	58M	114	476	1.1	100
5×10^{-7} M	58M	24	114	1.0	0

Table V. *GC-MS-SIM Results of Fraction Groupings (Fig. 4) from Normal Phase [-N(Me)]$_3$ HPLC*

Data from apices of 58M at 14 d. Values for endogenous GAs have not been corrected for contributions from deuterated internal standards. All of the data in each pair of horizontal lines (intensities above and relative abundances immediately below) come from a single injection of the sample identified in the sample or standard column.

Standard or Sample	Ion (*m/z*) Intensities and Relative Abundances in ( )					
	$[^2H_2]GA_1$, KRI = 2672			GA_1, KRI = 2672		
	508	*493*	*450*	*506*	*491*	*448*
Standard, $[^2H_2]$-GA_1	5842 (100)	tr.[a]	2148 (37)	ND[b]	ND	ND
B3	7774 (100)	189 (2)	2944 (38)	1768 (100)	tr.	804 (45)
	$[^2H_2]GA_{20}$, KRI = 2493			GA_{20}, KRI = 2493		
	420	*405*	*377*	*418*	*403*	*375*
Standard, $[^2H_2]$-GA_{20}	355489 (79)	63905 (14)	448036 (100)	ND	2076 (0.5)	3546 (0.8)
C2	52450 (43)	6998 (6)	121525 (100)	17521 (45)	3208 (8)	38792 (100)
	$[^2H_2]GA_{19}$, KRI = 2602			GA_{19}, KRI = 2602		
	464	*436*	*376*	*462*	*434*	*374*
Standard, $[^2H_2]$-GA_{19}	993 (3)	40131 (100)	33569 (84)	ND	1494 (4)	2835 (7)
C3	525 (3)	15444 (100)	16286 (105)	114 (1)	18581 (100)	13975 (75)
	$[^2H_2]GA_{53}$, KRI = 2504			GA_{53}, KRI = 2504		
	450	*418*	*391*	*448*	*416*	*389*
Standard, $[^2H_2]$ GA_{53}	26813 (100)	8111 (30)	21791 (81)	ND	ND	2996 (11)
D	9719 (100)	60 (1)	6904 (71)	528 (100)	ND	1495 (283)
	$[^2H_2]/GA_{20}$, KRI = 2491		GA_{44}, KRI = 2794			GA_{17}, KRI = 2581		
	420	*377*	*432*	*417*	*373*	*492*	*460*	*432*
Standard, $[^2H_2]$ GA_{20}	101883 (52)	195929 (100)	ND	ND	ND	ND	ND	ND
C1	61300 (52)	117775 (100)	3058 (100)	tr.	1211 (40)	2049 (73)	2810 (100)	1992 (71)

[a] Peak evident but too small for proper integration. [b] Not detected.

Table VI. *GC-MS-SIM Analysis of Putative GA_3 in Sorghum Leaf Extracts with $[^2H_2]GA_3$*

The fraction was equivalent to B3 in Figure 3. KRI for both samples was 2692.

Sample	Ions		
	m/z (relative abundance)		
58M leaves, day 12	504 (100)	489 (9.2)	370 (10.5)
$[^2H_2]GA_3$	506 (100)	491 (5.8)	372 (10.2)

To determine whether concentrations of GAs at 14 d are typical for the seedling stage of growth, growth and GA concentrations were determined in 7-, 10-, 12-, and 14-d-old plants (plants grown at different times). GA_1 concentrations for whole plants remained about twofold higher in 58M than the other two genotypes for the 7-d period (Fig. 4; Table IX). Similar trends were seen for GA_3 concentrations; however, these estimates are tentative for reasons discussed previously (Table VIII).

Comparing different GAs, the highest concentrations occurred in the GA_{19} and GA_{53} pools (Tables VIII and IX). This suggests that conversion of GA_{53} to GA_{44} and GA_{19} to GA_{20} may be the major rate limiting steps in the pathway leading to GA_1 and GA_3.

Since a plant symptomatic of elevated GA activity might also be the result of increased sensitivity to endogenous GAs because of its concentrations of IAA or ABA (3, 10), we analyzed those substances in 14-d-old plants (Table X). ABA concentrations varied from about 40 to about 80 ng g dry weight and IAA concentrations ranged from about 100 to about 210 ng g dry weight. In either case there was not a consistent pattern between the three genotypes. On the basis of these data we conclude that 58M does not differ significantly in ABA or in IAA concentrations from 90M and 100M.

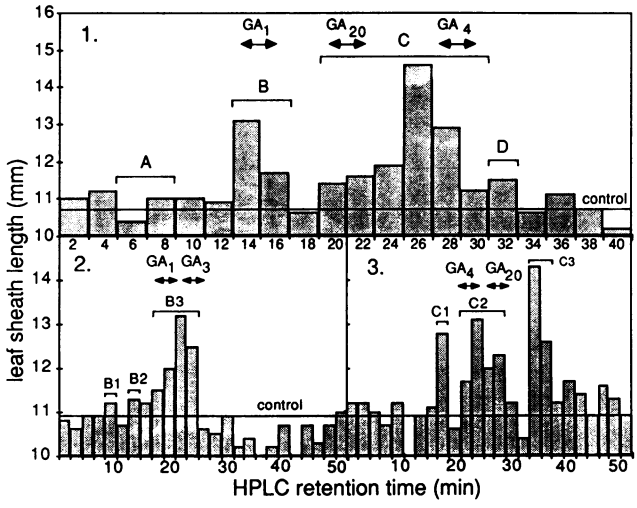

Figure 3. Dwarf rice leaf sheath growth in response to HPLC factions of extracts of sorghum plants. Panel 1 is for C_{18} HPLC and panels 2 and 3 are for Nucleosil $N(Me)_2$ HPLC of fractions B and C from panel 1. Data are from 58M culms at 14 d but are similar in pattern to those for all genotypes, tissues, and dates. Elution of authentic GA standards are indicated on the figure.

Table VII. *Distribution of Biological Activity from Extracts of 14-d-old Sorghum Plants*

Plants were separated into the indicated parts at the time of harvest before being freeze-dried. Extracts were fractionated by reverse-phase HPLC and biological activity detected with the dwarf-rice bioassay in serial dilution. Values, in ng GA_3 equivalents, were calculated from the dilution on the bioassay giving the greatest activity and corrected for dry weight and recovery of the internal standards added at the beginning of the extractions. Bioactivity designated as mono-OH is that eluting in fractions 20-32 from HPLC and di-OH as that in fractions 8-18 except for that coeluting with the $[^3H]GA_1$ internal standard and labeled as GA_1, with the remaining biological activity being 'other'. The precursors and conjugates fractions did not contain bioactivity for any genotype or tissue.

Tissue/ Genotype	Total Free Acids	mono-OH	di-OH	GA_1	Other
	ng GA_3 equivalents/gm dry wt				
Apices					
100M	137.6	93.9	14.8	16.3	12.6
90M	148.0	84.4	26.8	10.3	26.5
58M	299.9	151.0	70.5	56.7	21.7
Culms					
100M	114.2	95.2	9.6	3.3	6.1
90M	55.8	44.0	ND[a]	8.1	3.7
58M	81.6	60.0	4.1	14.2	3.3
Leaves					
100M	21.5	11.5	1.6	8.0	0.4
90M	15.0	9.6	1.1	1.3	3.0
58M	43.5	16.8	3.9	20.3	2.5

[a] Not detected.

DISCUSSION

Under high light intensity and elevated CO_2 level in the environmental chambers employed in this study, the genetic potential for ma_3^R-containing genotypes to grow faster than non-ma_3^R genotypes was demonstrated. 58M clearly increased in leaf sheath length, leaf blade length, total height, and dry weight more rapidly than 90M and 100M (Fig. 1; Table I). Since these genotypes differ only in the presence or absence of the mutant ma_3^R allele, it is apparent that this allele promotes growth rate as well as hastening the time to floral initiation. Quinby (19) had noted earlier that maturity genes influenced both growth rate and flowering date, but the enhanced accumulation of dry matter in the $ma3^R$-containing genotypes had not been detected previously (15).

The ma_3^R-containing genotype is GA_3 responsive, expressing increased shoot elongation (Table II) and hastening of floral initiation (Table III) in response to treatment. In addition, exposure of 58M to tetcyclacis, which presumably would lower the endogenous GA levels, shortened leaf sheaths, increased tillering, and delayed floral initiation (Table IV). The result was a 58M plant made phenotypically more like 90M and 100M. Previously, non-ma_3^R-containing genotypes had been made ma_3^R-like by treatment with GA_3 (16), and that response repeated here (Table II). In view of these results, 58M apparently is not a GA response mutant, and its morphology and flowering are manipulated by a presumed reduction in GA concentration by tetcyclacis.

At 14 d the levels of GA_1 were up to sixfold higher in apices

Table VIII. *Endogenous GA Content in 14 d-old Sorghum Plants*

GA content was measured by GC-SIM with $^2H_2$-labeled internal standards for the individual GAs, except for GA_3, which was measured against $[^2H_2]GA_1$, and GA_{44} and GA_{17}, which were measured against $[^2H_2]GA_{20}$. All values have been corrected for the recovery of the $[^3H]$GAs used as internal standards during extraction and purification.

Tissue	Endogenous GA Content						
	GA_1	GA_3	GA_{20}	GA_{19}	GA_{17}	GA_{44}	GA_{53}
	ng g^{-1} dry wt (ng GA/plant part)						
Apices							
58M	33.8	7.6	32.6	258.8	ND[a]	29.5	113.1
	(0.3)	(0.1)	(0.3)	(2.3)		(0.3)	(1.0)
90M	10.3	7.7	4.1	171.0	ND	7.0	55.4
	(0.1)	(0.1)	(0.04)	(1.7)		(0.1)	(0.6)
100M	1.4	12.7	13.3	286.4	ND	7.4	ND
	(0.01)	(0.1)	(0.1)	(2.9)		(0.1)	
Culms							
58M	32.1	6.8	11.1	215.0	6.3	9.3	10.0
	(2.3)	(0.5)	(0.8)	(15.4)	(0.5)	(0.7)	(0.7)
90M	26.7	tr.[b]	17.4	129.4	NA[c]	NA	154.2
	(0.9)		(0.6)	(4.6)			(5.4)
100M	25.9	ND	18.3	81.1	ND	1.2	166.2
	(0.9)		(0.7)	(2.9)		(0.04)	(6.0)
Leaves							
58M	20.5	26.0	16.7	34.7	3.9	tr.	ND
	(3.0)	(3.8)	(2.4)	(5.0)	(0.6)		
90M	ND	ND	12.4	22.9	1.5	tr.	NA
			(1.4)	(2.6)	(0.2)		
100M	14.1	10.8	21.5	69.2	2.4	2.0	NA
	(1.7)	(1.3)	(2.6)	(8.3)	(0.3)	(0.2)	

[a] Not detected. [b] Trace, peaks too small for accurate integration. [c] Not analyzed.

of 58M plants than the average for 90M and 100M and twofold higher in leaf blade tissue (Table VIII). The differences are not quite as large for bioassay activity, but 58M also had twofold or higher levels of bioactivity in apices and leaves (Table VII). Thus, it appears possible that the ma_3^R allele enhances the levels of biologically active GA in early vegetative stages and thereby promotes rapid shoot development and dry matter accumulation. Rood *et al.* (27) have noted that elevated GA levels correlate with increased growth rates of maize hybrids above growth rates of parental lines and proposed that hybrid vigor may be partially accounted for by elevated GA levels. The higher GA levels and growth rates in 58M are consistent with their hypothesis.

It should be noted that there are also major effects of ma_3^R on development. Since 58M initiates a floral meristem which terminates vegetative development around 20 d and 90M and 100M initiate around 50 d, at maturity the former has fewer leaves, fewer internodes, less dry weight and height than the latter. All of these differences are readily apparent in field plants (data not given). 90M and 100M exhibited more tillering (Table IV) and initiated more adventitious roots than 58M, and we have previously noted that treatment of non-ma_3^R sorghum genotypes with GA_3 markedly reduces both tillering and adventitious root development (12, 15). These observations are also consistent with 58M being elevated in GA levels and thus not tillering to the degree that non-ma_3^R genotypes do.

The early 13-hydroxylation pathway of GA biosynthesis has been established as the major pathway in many plant species, with the sequence $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20} \rightarrow GA_1$ (17, 28). GA_1 appeared to be the only GA in the pathway which is active in shoot elongation in maize, rice, and peas (17, 28). More recently (4), GA_{20} has been shown to yield both GA_1 and GA_5 with GA_5 being converted to GA_3 but the GA_1:GA_3 ratio is 50 to 1 or greater. Rood *et al.* (25) identified GA_1, GA_{19}, and GA_{20} in shoot cylinders of 45-d-old field-grown sorghum plants. Our data (Fig. 3; Table V) confirm the presence of GA_1, GA_{19}, and GA_{20} in sorghum and, in addition, we identified GA_{53} and GA_3 and tentatively identified GA_{44} and GA_{17}, an inactivation product of GA_{19} (17). Because we bioassayed the entire range where free GAs would normally come off of a C_{18} column (Fig. 3) and identified the major GA in each major peak of biological activity (Fig. 3; Table V), it seems very likely that the early 13-hydroxylation pathway produces the predominant GAs active in shoot elongation in vegetative sorghum plants.

Whether the elevated concentrations of GA_1 in 58M are sufficient to produce the longer leaf sheath and blades, reduced tillering, earlier flower initiation, and other phenotypic characteristics of the ma_3^R allele remains to be established. The morphology of grass seedlings is not convenient for isolation of organs; in these experiments the 'apices' sample contained the apical meristem, compressed nodes and internodes and the base of the leaf sheaths of all leaves (Fig. 1). The 'culm' sample contained all leaf sheaths of expanded leaves and the blades of all leaves partially elongated but not

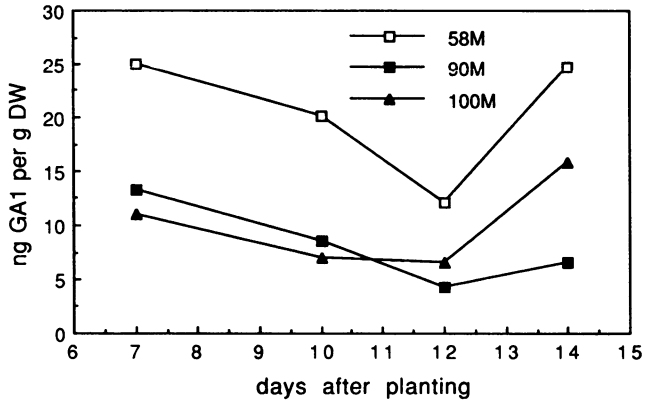

Figure 4. Time course of levels of GA_1 in whole shoots of sorghum seedlings. Plants for different age groups grown at different times.

yet exhibiting a leaf collar. In contrast, the 'leaf' sample contained leaf blade tissue which had mostly stopped or slowed in growth. To answer more adequately the question of whether the ma_3^R allele promotes growth by promoting GA_1 concentrations, it will be necessary to isolate samples containing rapidly expanding leaf blades and sheaths (which are enclosed inside of the culm) and determine both growth rates and GA_1 contents.

58M is one of the first GA-overproducing mutants so identified. 'Slender' (tall) mutants of peas, barley, and tomatoes are known. Slender pea (*la cry*^{s}) is insensitive to GA_3, growth retardants, and the na gene which severely reduces endogenous GA levels; it contains lower levels of GA-like substances than dwarfs which are *La Cry*^{s} (18). Slender barley (*sln* 1) is insensitive to growth retardants and contains no more GA activity than the wild type (11). Slender tomato (*pro*) contains less GA than its wild-type parent (GA_{20} and GA_1 assayed by GC-MS) (7), but it exhibits additional growth in response to GA_3 (6, 7). Tall mutant *gigas* barley exhibits reduced growth in response to CCC and a GA-depleting dwarfing gene (Fauret *et al.*, cited in ref. 22). Another tall mutant of pea (*lv*) has been reported to have increased sensitivity to applied GA_1 but to have normal synthesis and metabolism of GAs (23). None of the tall mutants studied in detail appear to be GA-overproducers with the possible exception of *gigas* barley (22). While our work has been progressing, Rood *et al.* (26) have characterized a tall, canola mutant, which overproduces GA_1 and GA_3 (to a greater degree).

Table IX. *Endogenous GA Content of Sorghum Plants*

GA content measured by GC-MS-SIM using deuterated internal standards. Values for 7 d and 10 d plants were derived from the analysis of whole shoots, while those from 14 d plants were summed from separate measurements of apices, leaves, and culms. All values have been corrected for recovery of [^{3}H]GAs used as extraction and chromatographic standards and for differences in dry weight.

Sample	GA Concentration in Sorghum Shoots			
	GA_1	GA_3	GA_{20}	GA_{19}
	ng g^{-1} dry wt			
Day 7				
58M	25.0	21.3	55.7	15.4
90M	13.3	8.5	28.2	23.8
100M	11.0	23.4	33.7	20.6
Day 10				
58M	20.2	8.9	52.8	7.9
90M	8.5	4.5	37.1	15.9
100M	7.0	6.5	34.4	13.3
Day 14				
58M	24.7	19.2	15.5	100.7
90M	6.6	0.5	13.0	55.9
100M	15.9	8.6	20.3	85.1

Table X. *Endogenous IAA and ABA Content of 14 d-old Sorghum Plants*

Plants were separated into the indicated parts (see Fig. 1) at the time of harvest and lyophilized. IAA and ABA content was measured by GC-MS-SIM using [$^{13}C_6$]IAA and [$^2H_6$]ABA as internal standards. All values have been adjusted for dry weight.

Sample	ABA	IAA
	ng g^{-1} dry wt	
Apices		
58M	38.4	173
90M	85.3	106
100M	58.0	208
Culms		
58M	72.0	138
90M	42.4	123
100M	41.6	124
Leaves		
58M	79.2	213
90M	49.2	115
100M	57.6	167

It should be noted that the slender mutations in pea, tomato, and barley all modify multiple characters in addition to internode length. The *pro* tomato has altered leaf shapes and fewer adventitious roots (6). Slender pea exhibits rapid seed germination, pale foliage, reduced branching, malformed and abortive flowers, reduced seed set, or parthenocarpic pods (18). In both cases application of GA_3 to the wild type can produce phenocopies of the mutants. The mature slender barley plant is limber and exhibits increased basal elongation and tillering, root initials on lower nodes, narrow leaves, longer than normal heads, and flowers which are sterile (11). As discussed above, the ma_3^R allele in sorghum also produces multiple phenological changes, and treatment of the wild type with GA_3 duplicates the ma_3^R phenology (16).

DEDICATION

This manuscript is dedicated to the memory of J. Roy Quinby, 1901–1988, whose vision, insight, and persistence led to recognition of the significance of the maturity genes in sorghum and their collection and preservation for contemporary studies.

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