Plant Physiol. (1995) 108: 337–343

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

IX. The ma_3^R Allele Disrupts Diurnal Control of Gibberellin Biosynthesis

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The diurnal regulation of gibberellin (GA) concentrations in *Sorghum bicolor* was studied in a mutant lacking a light-stable 123-kD phytochrome ($ma_3^Rma_3^R$), wild-type (ma_3ma_3,Ma_3Ma_3), and heterozygous ($ma_3ma_3^R$) cultivars. GAs were determined in shoots of 14-d-old plants by gas chromatography-selected ion-monitoring-mass spectrometry. GA_{12} levels fluctuated rhythmically in Ma_3Ma_3, ma_3ma_3, and $ma_3^Rma_3^R$; peak levels occurred 3 to 9 h after lights-on. In some experiments, GA_{53} levels followed a similar pattern. There was no rhythmicity in levels of GA_{19} and GA_8 in any genotype. In ma_3ma_3 and Ma_3Ma_3, GA_{20} levels increased at lights-on, peaked in the afternoon, and decreased to minimum levels in darkness. In $ma_3^Rma_3^R$, peak GA_{20} levels occurred at lights-on, 9 h earlier than in the wild-type genotypes. The pattern for GA_1 levels closely followed GA_{20} levels in all cultivars. One copy of ma_3 restored near wild-type regulation of GA_{20} levels. GA rhythms persisted in 25-d-old ma_3ma_3 plants. Since absence of the 123-kD phytochrome disrupted diurnal regulation of the $GA_{19} \rightarrow GA_{20}$ step, the $ma_3^Rma_3^R$ genotype may be viewed as being phase shifted in the rhythmic levels of GA_{20} and GA_1 rather than as simply overproducing them.

Many plant responses to environmental signals involve changes in hormone physiology (Evans, 1984; Vince-Prue, 1985), and in this way the environmental stimulus is translated into a physiological response. Since the phytochrome family of photoreceptors is involved in light perception (Sharrock and Quail, 1989; Smith and Whitelam, 1990), and bolting and flowering are thought to be regulated primarily by GAs (Pharis and King, 1985; Talon and Zeevaart, 1990; Wilson et al., 1992; Zeevaart and Gage, 1993; Zeevaart et al., 1993), a description of the interrelationships between these two regulatory chemical families is an important goal toward understanding the processes of photomorphogenesis.

GA biosynthesis is regulated by light. Photoregulation of GA metabolism may be the result of the presence or absence of light, the transition between light and dark, the duration or alteration of the photoperiod length, the quality of the light (particularly red and far red), and the quantity of light. Each has received separate attention, and the integration of these investigations is necessary to understand the role of the light environment on regulation of GA metabolism and, hence, of plant growth and development. In sorghum (*Sorghum bicolor* [L.]), as in many other plant species, GAs are produced via the early C-13-hydroxylation pathway, in the progression $GA_{12} \rightarrow GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20} \rightarrow GA_1 \rightarrow GA_8$ (Rood et al., 1986; Beall et al., 1991). GA_1 is the GA primarily responsible for regulation of vegetative growth in many species (Phinney et al., 1985; Ingram et al., 1986).

Both LDP and SDP species appear to react to changes in the light environment with changes in GA metabolism, and these changes are often correlated with changes in plant growth (i.e. bolting) and development (flowering). One of the fundamental problems in plant physiology is to reconcile how the transition between vegetative and reproductive growth in both SD and LD species is regulated by the metabolism (biosynthesis, conjugation, degradation, transport) of GAs in response to the opposite signals of long days and short days. However, until more is known about how light affects GA metabolism in each of these classes of plants, it will be difficult to discern how alterations in levels or patterns of GA metabolism are involved in photomorphogenesis.

Several of the steps in the early C-13-hydroxylation pathway have been reported to be regulated by the light environment, including steps upstream of GA_{12} (Zeevaart and Gage, 1993), $GA_{12} \rightarrow GA_{53}$ (Davies et al., 1986), $GA_{53} \rightarrow GA_{44}$ (Gilmour et al., 1986), $GA_{19} \rightarrow GA_{20}$ (Metzger and Zeevaart, 1980; Gianfagna et al., 1983; Gilmour et al., 1986; Zeevaart et al., 1993), and $GA_{20} \rightarrow GA_1$ (Lance et al., 1976; Campell and Bonner, 1986). The sorghum mutant ma_3^R lacks a 123-kD phytochrome (Childs et al., 1991, 1992) and is relatively insensitive to the photoperiod, flowering early in a range of photoperiods (Pao and Morgan, 1986). This mutant has been classified, based on sampling done at one time during the photoperiod, as a GA overproducer (Beall et al., 1991; Foster et al., 1994).

This sorghum system provides us with an excellent opportunity to examine the diurnal levels of GAs in a SDP and to determine whether GAs are synthesized in a rhythmic pattern under SD conditions, as was found for LD spinach (Talon et al., 1991). The phytochrome-deficient sorghum $ma_3^Rma_3^R$ genotype also provides an opportunity to test the hypothesis that the absence of the 123-kD phy-

[1] Supported by U.S. Department of Agriculture competitive grant 91-37304-6582 (P.W.M.) and a Postdoctoral Fellowship from the Natural Sciences and Engineering Research Council of Canada (K.R.F.).

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tochrome causes aberrant perception of the light environment, which disrupts control of GA biosynthesis.

MATERIALS AND METHODS

Plant Material

Sorghum (*Sorghum bicolor* [L.] Moench) plants of the genotypes Ma_3Ma_3 (100M), ma_3ma_3 (90M), and $ma_3^Rma_3^R$ (58M) were grown under a 12-h photoperiod, with 32/22°C day/night temperatures. Other growth conditions have been described (Beall et al., 1991). Harvests commenced at the beginning of the light period 14 d after seeding. Samples were collected at 3-h intervals until lights-off on d 15. At the transitions between lights-on and lights-off, samples were taken immediately before lights-off (at the end of the photoperiod) and immediately after lights-on at the beginning of the next photoperiod. For harvests during the dark period, plants were removed from the growth chamber in complete darkness and cut from the pots under a dim green safelight. At the time of harvest, the roots and two basal leaves and sheaths were removed; the shoot was cut at the collar of the tallest leaf sheath, and the leaf blades were discarded. The samples were frozen in liquid N_2 within 20 min following the removal of the plants from the growth chamber. After lyophilization, the third leaf blade and sheath were removed, leaving a shoot sample containing the youngest, most actively elongating tissues from 30 to 60 plants. The samples were stored at −70°C until extracted for GAs.

The heterozygous genotype $ma_3ma_3^R$, resulting from the cross of cytoplasmically male-sterile $A_3$90M with 58M, was grown and harvested as described above. $A_3$90M was generated from the original cross of A_3Tx430 × 90M, followed by five backcrosses with selection at nurseries in College Station, TX, and Puerto Rico. Heterozygous $ma_3ma_3^R$ was then generated by crossing $A_3$90M with 58M. This genotype was used to determine whether the effect of one copy of ma_3^R was sufficient to disrupt GA biosynthesis.

At the present time, we are conducting an investigation of the effect of photoperiod length on GA metabolism in older sorghum plants. A subset of these trials is relevant to the present communication. Plants having the wild-type genotype ma_3ma_3 (90M) were grown until they were 25 d old and then harvested at 4-h intervals throughout a 12-h photoperiod. Following lyophilization and removal of the basal four leaves and sheaths, the samples were extracted and analyzed for GAs as described above. Each sample included the shoots of 15 to 20 plants. Only one sample was obtained at each harvest time.

GA Extraction, Purification, and Analysis

GAs were extracted in methanol and purified using reverse and normal phase preparatory chromatography, partitioned (using aqueous buffer, ethyl acetate, and ether phases), and chromatographed (HPLC) as described by Foster et al. (1994), except that the samples were not purified using amino extraction cartridges prior to HPLC. GAs were derivatized and analyzed by GC-single ion-monitoring-MS as described by Beall et al. (1991) and Foster et al. (1994). Deuterated internal standards (25 ng each of $[^2H_2]GA_1$, $[^2H_2]GA_8$, $[^2H_2]GA_{19}$, $[^2H_2]GA_{20}$, and $[^2H_2]GA_{53}$ and 20 ng of $[^2H_2]GA_{12}$) were added to the extracts following methanolic extraction. Tritiated standards (1500 Bq each of $[^3H]GA_1$ and $[^3H]GA_4$) were also added to monitor recovery throughout the purification procedure.

Differences in GA content between the dominant wild-type 100M sorghum (Ma_3Ma_3) and phytochrome-deficient 58M sorghum ($ma_3^Rma_3^R$) were a primary interest. Therefore, a major effort was devoted to analyzing these genotypes. Two preliminary experiments using 100M were conducted, with the first yielding results only for GA_{20} and the second yielding results for GA_{19}, GA_{20}, and GA_1. A third 100M experiment included the collection of three replicate samples at each harvest time. Data for GA_{12}, GA_{53}, GA_{19}, GA_{20}, GA_1, and GA_8 were obtained from this sample set. For 58M, two experiments were conducted; a single sample was collected at each harvest time in the preliminary experiment, and three replicate samples were collected in the second. GA_{12} data were obtained in only the unreplicated experiment, whereas GA_{53}, GA_{19}, GA_{20}, GA_1, and GA_8 data were obtained from both trials. Since the growth and development of ma_3ma_3 (90M) is very similar to Ma_3Ma_3 (100M), a fully replicated analysis of GA levels in 90M was not warranted. Only one 90M experiment was conducted, without replication, to confirm similarity between Ma_3 and ma_3. Because of limited seed availability, only a single heterozygote ($ma_3ma_3^R$) experiment could be conducted, with three replicates collected per sample time.

While these analyses were being conducted, GA_{12} was occasionally absent from many of the samples within each sample set. When this occurred the complete GA_{12} data set was considered invalid, and these data are not reported (e.g. the replicated 58M trial, the unreplicated 100M trials, and the $ma_3ma_3^R$ experiment). The loss of GA_{12} was found to occur during C_{18} reverse-phase preparatory chromatography if the extract was at or below pH 7. The extracts from larger samples (>1 g dry weight) were below pH 7, resulting in the retention of GA_{12} on the C_{18} column packing. GA_{12} was recovered when extract pH was adjusted to pH 8 (using 2 N NH_4OH) prior to C_{18} preparatory chromatography. Sample pH did not affect recovery of the other GAs.

RESULTS AND DISCUSSION

The data presented in Figures 1, 2, and 3 provide evidence for diurnal regulation of GA_{12} levels in 14-d-old sorghum. The ma_3^R mutation had no effect on the levels or rhythmicity of production of GA_{12}, since the amount and timing of maximum and minimum levels of GA_{12} were similar among non-ma_3^R and ma_3^R genotypes. GA_{12} levels increased from lights-on through to late in the photoperiod, with maximum levels of 7 to 12 ng g^{-1} dry weight observed 3 h prior to lights-off in these three genotypes. The concentration of GA_{12} diminished during the last 3 h of the light period (5–8 PM) of both days in 90M and 100M and of the 2nd d in 58M. Nighttime GA_{12} levels were about half those observed during the light periods. These data do not indicate whether diurnal control occurs at GA_{12} synthesis or upstream of GA_{12}. Zeevaart and Gage (1993) have dem-

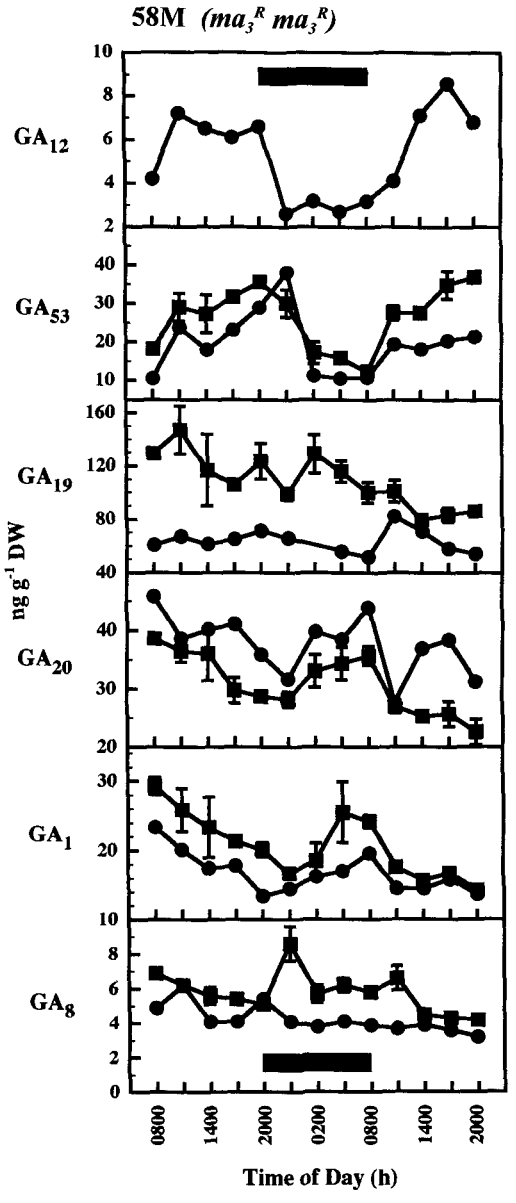

Figure 1. Diurnal regulation of GA levels in shoots of 14-d-old seedlings of 58M, a phytochrome-deficient sorghum mutant ($ma_3^Rma_3^R$). Plants were grown in a 12-h photoperiod. The dark period is indicated by the solid bars at the top and bottom of the figure. GA levels were measured by GC-selected ion-monitoring-MS using deuterated internal standards. In the first experiment (●), a single sample was obtained at each harvest time. In the second (■), data are the means of three replicate samples that were obtained at each harvest time. In this experiment, the SEs of the means are indicated for each time; where absent, they are hidden within the symbol. DW, Dry weight.

onstrated photoperiodic control of *ent*-kaurene biosynthesis in two LD species, and it is conceivable that similar controls operate in SD sorghum. Direct evidence of phytochrome participation in the control of GA production at the level of *ent*-kaurene synthesis is lacking. However, if a phytochrome is involved at this level, it is unlikely to be the 123-kD phytochrome lacking in ma_3^R plants (Childs et al., 1991, 1992; Foster et al., 1994), since similar levels and diurnal rhythmicity of GA_{12} levels were found in $ma_3^Rma_3^R$, ma_3ma_3, and Ma_3Ma_3.

In both $ma_3^Rma_3^R$ experiments (Fig. 1) and in the replicated Ma_3Ma_3 experiment (Fig. 3), levels of GA_{53} increased immediately following lights-on at 8 AM, peaked late in the day, and decreased following lights-off at 8 PM. This pattern is similar to that observed for GA_{12} and suggests that GA_{53} levels are determined by the level of its precursor,

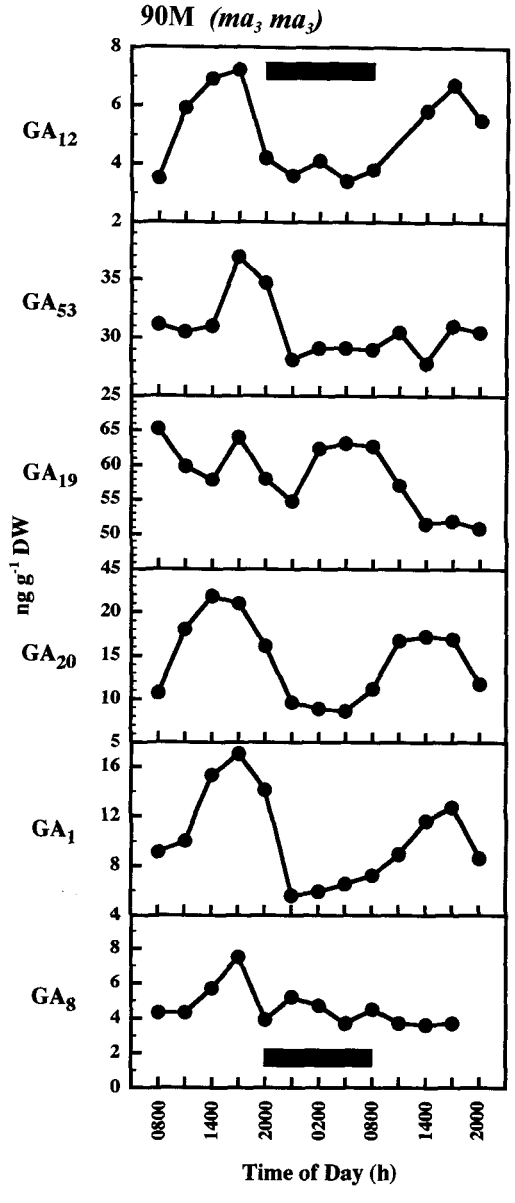

Figure 2. Diurnal regulation of GA levels in shoots of 14-d-old seedlings of 90M wild-type sorghum (ma_3ma_3). Plants were grown in a 12-h photoperiod. The dark period is indicated by the solid bars at the top and bottom of the figure. GA levels were measured by GC-selected ion-monitoring-MS using deuterated internal standards. Data are the results of a single sample obtained at each harvest time. DW, Dry weight.

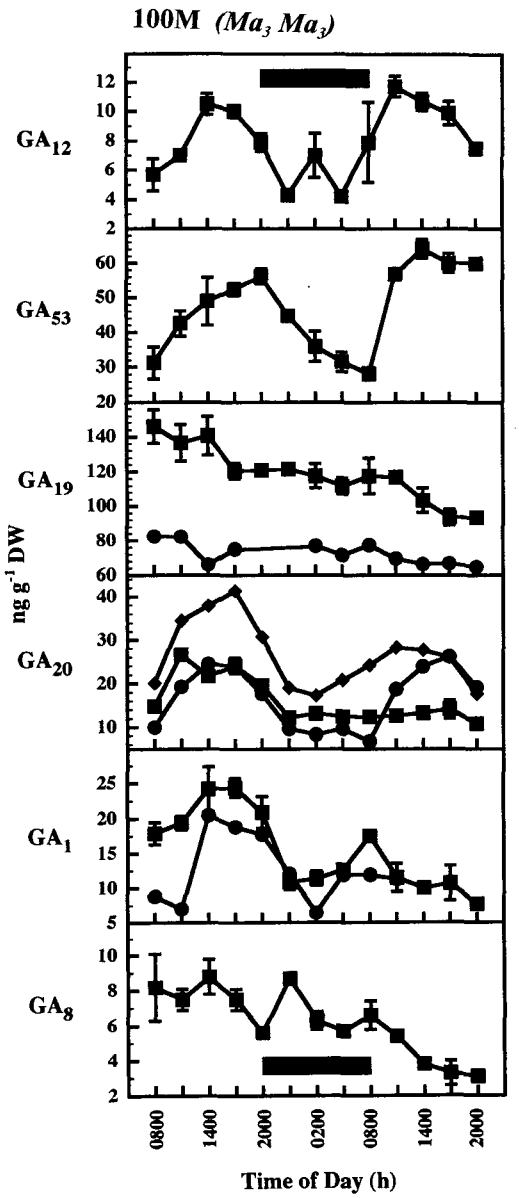

Figure 3. Diurnal regulation of GA levels in shoots of 14-d-old seedlings of 100M wild-type sorghum (Ma_3Ma_3). Plants were grown in a 12-h photoperiod. The dark period is indicated by the solid bars at the top and bottom of the figure. GA levels were measured by GC-selected ion-monitoring-MS using deuterated internal standards. A preliminary experiment yielded results only for GA_{20} (♦). A single sample was harvested at each time in the second experiment (●) and yielded data for GA_{19}, GA_{20}, and GA_1. Three replicate samples were harvested at each time in the third experiment (■). In this experiment, the SEs of the means are indicated for each time; where absent, they are hidden within the symbol. DW, Dry weight.

GA_{12}. Since rhythmicity in GA_{53} levels was not clearly expressed in 90M and was absent in the heterozygote (Fig. 4), it is not possible to draw further conclusions regarding light effects on this step in the pathway.

There was no evidence of diurnal regulation of GA_{19} level in any of the genotypes (Figs. 1–4). GA_{53} oxidase ($GA_{53} \rightarrow GA_{44}$) activity was reported to be controlled by light (Gilmour et al., 1986); however, the absence of rhythmic alterations in the pattern of GA_{19} levels that correlated with the light regime suggests that in sorghum there is no effect of light on the $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19}$ portion of the pathway. It appears that the effect of the regulation of a particular step in the synthesis of GAs (i.e. control of GA_{12} levels) is lost as the distance from the point of control increases.

Spinach grown in short days produced GA_{20} from GA_{19} during the 8-h light period, and in spinach growing in long days (8 h of high-intensity light, 16 h of low-intensity light), GA_{20} levels increased during the 16-h extended day (Talon et al., 1991). In both light conditions, GA_{20} was produced rhythmically as evidenced by analysis of two harvests per

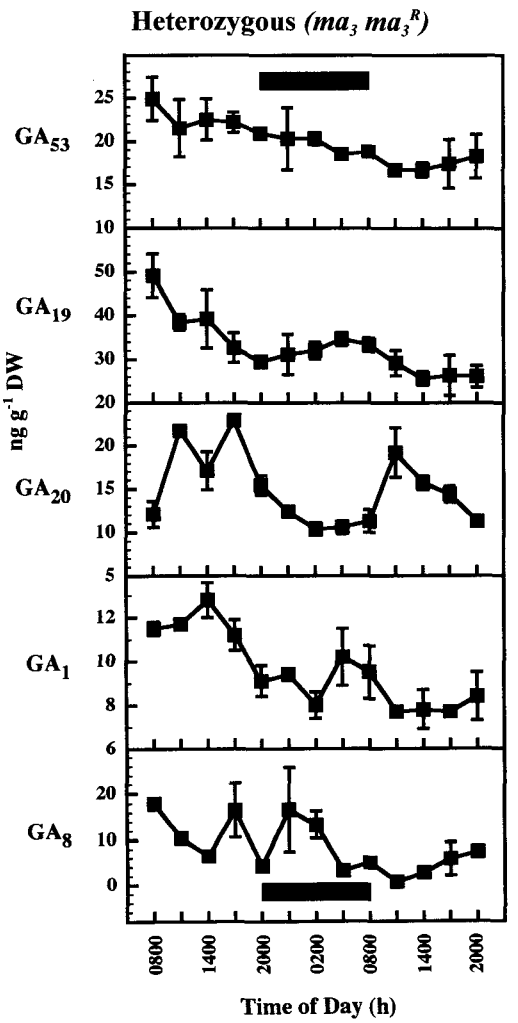

Figure 4. Diurnal regulation of GA levels in shoots of 14-d-old seedlings of heterozygous ($ma_3ma_3^R$) sorghum. Plants were grown in a 12-h photoperiod. The dark period is indicated by the solid bars at the top and bottom of the figure. GA levels were measured by GC-selected ion-monitoring-MS using deuterated internal standards. Data are the means of three replicate samples. The SEs of the means are indicated for each time; where absent, they are hidden within the symbol. DW, Dry weight.

24 h, and this reaction was considered to be under phytochrome control. In sorghum, the GA_{20} levels also appear to be regulated by light. GA_{20} levels increased during the early portion of the light period, peaked late in the 12-h day, and decreased to minimum levels during the dark period. In ma_3ma_3 (Fig. 2), the two unreplicated Ma_3Ma_3 trials (Fig. 3), and in $ma_3ma_3{}^R$ (Fig. 4), the cycle was repeated during the second light period, whereas in the replicated Ma_3Ma_3 trial (Fig. 3), this pattern was observed only during the first light period. Daytime maxima were generally 2-fold greater than the nighttime minima. Rhythmic production of GA_{20} in non-$ma_3{}^R$ sorghum grown under the noninductive 12-h photoperiod used in these experiments is, therefore, similar to that occurring in spinach grown under noninductive 8-h short days (Talon et al., 1991).

There was also a rhythm in the level of GA_{20} in $ma_3{}^Rma_3{}^R$, although the alteration in level was smaller than the 2-fold differences in the other genotypes. Also, this rhythm was phase shifted relative to ma_3ma_3 and Ma_3Ma_3. In $ma_3{}^Rma_3{}^R$, GA_{20} levels increased during the night, with the peak level occurring near lights-on (Fig. 1). Levels decreased during the light period to a minimum at, or just after, the end of the photoperiod. Absence of the 123-kD phytochrome in $ma_3{}^Rma_3{}^R$ thus results in disruption of normal diurnal control of synthesis of GA_{20} from GA_{19} (relative to the wild-type genotypes ma_3ma_3 and Ma_3Ma_3). In the case of spinach, Gilmour et al. (1986) and Talon et al. (1991) have provided evidence that GA_{19}-oxidase activity is present only in light and disappears after 2 to 3 h of darkness. However, the data presented here suggest that regulation of this metabolic step is more complicated, since in the absence of the 123-kD phytochrome (and presumably of any metabolic regulation imposed by this phytochrome), GA_{19}-oxidase was active in the dark. The similarity of the rhythm and levels of GA_{20} in the heterozygous $ma_3ma_3{}^R$ (Fig. 4) to those in ma_3ma_3 (Fig. 2) and Ma_3Ma_3 (Fig. 3) shows that one wild-type allele (in this case ma_3) restores wild-type diurnal regulation of GA_{20} biosynthesis.

Synthesis of GA_1 appears to occur rapidly from GA_{20}. In all genotypes, the pattern of GA_1 levels closely followed the pattern observed for GA_{20}, and absolute levels of GA_1 were slightly lower than those observed for GA_{20}. In $ma_3{}^Rma_3{}^R$ (Fig. 1), GA_1 peaked at or slightly before 8 AM (lights-on), whereas in ma_3ma_3 (Fig. 2) and Ma_3Ma_3 (Fig. 3) peaks occurred at 5 PM (9 h into the 12-h light period). GA_1 levels were highest in the heterozygous plants during the 1st d at 2 PM; however, no nighttime GA_1 minimum was observed; nor was there a peak during the 2nd d (Fig. 4). In all genotypes, levels of GA_8 were relatively constant (Figs. 1–4) and showed no pattern that correlated with the photoperiod or with the levels of any other GAs.

Rhythmic patterns in GA_{12}, GA_{20}, and GA_1 content were generally more strongly expressed during the 1st d than during the 2nd d (Figs. 1–4), raising questions about the persistence and reproducibility of the observed rhythms. A subset of data collected in a continuing investigation of GA levels in older ma_3ma_3 plants being conducted to answer other questions regarding GA metabolism and flowering is relevant here. An analysis of GA levels in older $ma_3{}^Rma_3{}^R$ plants was not appropriate, because in the 12-h photoperiod, these plants initiate flowering at 20 d. Therefore, any patterns observed in 25-d-old $ma_3{}^Rma_3{}^R$ would have been complicated by the possible changes in GA metabolism occurring as these plants entered the reproductive phase of growth.

Samples of 25-d-old ma_3ma_3 were obtained at 4-h intervals throughout the light period. GA_{12} and GA_{53} levels (Fig. 5) increased during the light period in a pattern similar to that observed in ma_3ma_3 and Ma_3Ma_3 (Figs. 2 and

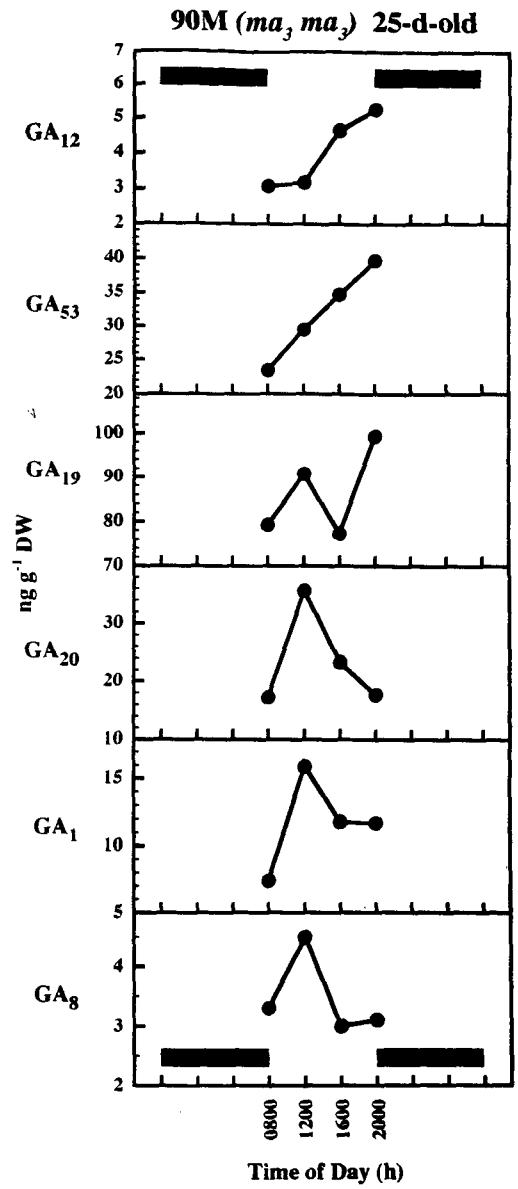

Figure 5. Levels of GA_{12}, GA_{53}, GA_{19}, GA_{20}, GA_1, and GA_8 in shoots of 25-d-old 90M (ma_3ma_3) sorghum plants. Plants were grown in a 12-h photoperiod. GA levels were measured by GC-selected ion-monitoring-MS using deuterated internal standards. Data are the results of a single sample obtained at 4-h intervals throughout the light period. DW, Dry weight.

3). GA_{19} levels did not follow a discernible rhythm in the older plants (Fig. 5). GA_{20} and GA_1 levels (Fig. 5) follow a pattern similar to that observed during the 1st d in younger sorghum plants (Figs. 2 and 3). In 25-d-old ma_3ma_3, levels of these GAs increased during the early portion of the light period and began to diminish late in the photoperiod (Fig. 5). These data strongly suggest that the rhythms in GA_{20} and GA_1 levels observed during the first light period in the non-ma_3^R experiments (Figs. 2 and 3) persist and can be observed in older plants as well as in 14-d-old plants. We have no data to explain the decrease in the GA peaks during the second light period in the younger plants; however, the decreased expression of the rhythm may be related to the frequent opening of the growth chamber during sampling or to a change in the light environment as a consequence of the thinning of the plant population with the progression of harvests. Plants perceive enrichment of far red light from adjacent vegetation, and extensive removal of pots of plants during our experiments may have altered the red:far red ratio perceived by the remaining plants (Ballaré et al., 1990; Smith et al., 1990). Plants harvested during the first 24 h of sampling were least disturbed.

That the absence of the 123-kD phytochrome alters, but does not remove, rhythmicity of GA_{20} and GA_1 levels in $ma_3^Rma_3^R$ is suggestive of other controls participating in GA_{20} synthesis. In the absence of this type II, light-stable phytochrome, a phytochrome subject to a different set of regulatory signals and differing in primary function may have some activity at the $GA_{19} \rightarrow GA_{20}$ step. This would explain the phase-shifted pattern observed in the GA_{20} and GA_1 levels in $ma_3^Rma_3^R$ relative to the other genotypes. Alternatively, there may be controls of GA_{20} biosynthesis that are in competition with that imposed by the 123-kD phytochrome, and in the absence of this phytochrome the control(s) exercised by the competitor(s) becomes visible. These highly speculative hypotheses await proper experimental analysis.

CONCLUSIONS

Two reactions in the biosynthesis of GAs in SD sorghum appear to be controlled by light in a diurnal rhythm, with phytochrome probably participating in regulation of both steps. The first is either synthesis of GA_{12} or, upstream of this step, possibly the synthesis of *ent*-kaurene (Zeevaart and Gage, 1993). This control is not imposed by the 123-kD phytochrome, since diurnal control of GA_{12} biosynthesis is evident in each genotype examined here (Ma_3Ma_3, ma_3ma_3, and $ma_3^Rma_3^R$). The second reaction is the synthesis of GA_{20} from GA_{19}. Since rhythmicity and absolute levels of GA_{20} production in the phytochrome-deficient genotype $ma_3^Rma_3^R$ are disrupted relative to the genotypes containing the wild-type alleles Ma_3 or ma_3 (and wild-type regulation of GA_{20} and GA_1 synthesis is restored in $ma_3ma_3^R$), we suggest that the 123-kD phytochrome, which is absent in $ma_3^Rma_3^R$, participates in the control of diurnal regulation of synthesis of GA_{20} and, hence GA_1, from GA_{19}. Confirmation of this conclusion awaits development of appropriate biochemical and molecular methods for the analysis of the interaction(s) of phytochrome with the enzyme(s) of this pathway.

The ma_3^R mutant has been classified as a GA overproducer, based on the analysis of sorghum samples collected during the first few hours following lights-on (Beall et al., 1991; Foster et al., 1994). Although at times during the 24-h cycle GA levels in ma_3^R plants exceeded those in non-ma_3^R sorghum, the results presented here demonstrate that the classification of ma_3^R as a GA overproducer is an oversimplification of the effect of the absence of the 123-kD phytochrome. ma_3^R may be more appropriately classified as a mutant that possesses aberrant perception of the light period, resulting in a modification in the timing of the production of GAs. Thus, as in LD spinach, the presence and timing of rhythmic pulses in the levels of GAs may play a role in the regulation of photoperiodic phenomena, including flowering, in SD species.

ACKNOWLEDGMENTS

We thank Dr. Frederick Miller, who supplied the seed used in this study and who developed the cytoplasmically male-sterile lines used to generate the $ma_3ma_3^R$ genotype. Deuterated GA standards were supplied by Dr. L. Mander, Australian National University, Canberra, Australia. We are grateful also to Ms. Michelle Chrisman, who assisted with the GA extraction and purification, and to Dr. Scott A. Finlayson who assisted with computer graphics.

Received November 3, 1994; accepted February 2, 1995.

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