THE MORPHOGENETIC EFFECTS OF THE HOODED GENE IN BARLEY II. CYTOLOGICAL AND ENVIRONMENTAL FACTORS AFFECTING GENE EXPRESSION[1]

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Received September 30, 1968

IN a previous publication (STEBBINS and YAGIL 1966), evidence was presented which indicates that the complex syndrome of morphological effects produced by the dominant gene hooded (*KK*) in barley is initiated by a more rapid proliferation of epidermal and subepidermal cells on the adaxial surface of the lemma primordium, when it is 200–250 micra long. In the present publication, the association of this more rapid proliferation with an increased frequency of nuclei in the S or synthetic stage of DNA replication will be established. In addition, some factors which can bring about phenotypic reversion toward the awned condition will be pointed out.

MATERIALS AND METHODS

The barley varieties used in this study were Atlas 46, Hooded Atlas 46, Atsel, and Hooded Atsel. These varieties, as well as the conditions under which they were grown, were described previously (STEBBINS and YAGIL 1966).

Determining the stages of development: At the stage when differentiation of lemma primordia takes place, the spike and lemma primordia are tightly enclosed in the developing culm leaves and their sheaths. If the latter are removed or in any way disturbed, development will not proceed normally. Consequently, an indirect method was devised for determining the developmental stage of the reproductive spike by observations of the developing leaves which surround it. This was possible because in the varieties used and under the constant environmental conditions employed for growing them, the developmental stage of the spike is closely correlated with that of the surrounding leaves. The predictive value of the leaf measurements employed was sufficiently high so that, within reasonable limits, we could estimate by them the developmental stage of the reproductive apex of the plants which were treated experimentally.

Cold treatment: Atsel Hooded plants were grown in vermiculite in a temperature regime of 21°C during a 16 hr daytime period and 17°C at night. At the stage when the apical meristems were entering the first stages of reproductive differentiation, successive groups of plants were transferred, at 2-day time intervals to a 16 hr illuminated cold room of 4.5°C. Each group of plants was kept under these conditions for 10 days. Each plant was labelled during the transfer according to its leaf development, as described above. After 10 days the plants were transferred back to the previous growth conditions until maturity.

Photoperiodic treatments: A long day consisted of 16 hr illumination and 8 hr darkness. A short day was 8 hr illumination and 16 hr darkness. These photoperiods were maintained either in the growth chambers or in the greenhouse. In the latter, long days consisting of natural daylight were supplemented during the evenings with incandescent illumination. Short day treated

[1] This research was supported in part by National Science Foundation Grant no. GB 2276. Taken in part from a dissertation submitted to the University of California in partial fulfillment of the requirements for the Ph.D. degree.

plants were covered daily with a hood of black material after the eight-hour daylight period. An interrupted night consisted of a short day photo-period with the night being interrupted after 7 hrs by a 20 min illumination period with a 60W incandescent bulb.

Production of heterozygous seeds: Crosses were made between Atlas Awned and Atlas Hooded genotypes, the latter being used as the pollen parent (Hooded is dominant to awned). The awns and about one-third of the lemma of the female parent were cut with a pair of scissors when the spike was ready to emerge from the sheath of the last leaf. The immature anthers were removed with a pair of fine forceps and the spikes were covered with aluminum foil. The desired pollen was applied 1–3 days later by shaking the paternal spike over the maternal one. All F_1 plants were of the hooded phenotype, indicating a 100% success of the method used.

Autoradiography: Tritiated thymidine (^{3}H-T) was used as a radioactively labelled precursor of DNA synthesis. Tritiated cytidine (^{3}H-C) with an oxy-ribose sugar moiety was used as precursor of RNA synthesis. Both radioactive compounds were obtained from Schwartz Bioresearch Inc.

Plants were excised and all their leaves, including the meristematic ones, were removed until the apical meristem was exposed. The meristematic apices were excised so that a few stem internodes were attached to them. They were then immersed in the radioactive solution (diluted to 5 μc/ml in a 2% sucrose solution). The treatment with ^{3}H-T lasted for 6 hrs and was followed by a 24 hr period in 2% sucrose solution. With ^{3}H-C the meristems were treated for 12 hrs in radioactive solution only. Following treatment, the apices were fixed in Carnoy's fixative, dehydrated, embedded and sectioned 8 μ thick. The sections were treated for 20 min with 2% perchloric acid and were then covered with Kodak AR-10 autoradiographic stripping film. After drying they were stored in the refrigerator. Exposure period lasted 14 days, after which the sections were developed in a Kodak D 19 b developer, fixed, washed and stained with Harris' hematoxylin (HUMASON 1962).

In ^{3}H-T-treated sections the percent of labelled nuclei was scored in the region of the lemma from which the hooded structure develops and in a comparable region in the awned genotype. Each count is the average of three successive sections. In ^{3}H-C-treated lemmas the number of grains over a cytoplasmic area of 108.17 μ^2 was conducted. Counts over nuclei were avoided. With both nucleotides the background label was negligible.

RESULTS

Cold treatment experiments: Atsel Hooded plants, when grown under the low temperature regime (4.5°C) for ten days, flowered 10–14 days later than the controls. In them, a small proportion of the florets developed, instead of normal hoods, awn-like structures or "Awn-phenocopies" (Figure 1). The development of the whole spike was weaker and about 20% of the plants died before maturity.

At the beginning of the cold treatment, the leaf development of each plant was recorded as described in the MATERIALS AND METHODS section in order to estimate the developmental stage of the apical meristem. In Figure 2 the results obtained from two experiments are graphically represented. This graph shows that the response to the cold treatment occurred only in plants subjected to the treatment at a stage when the fourth leaf was fully developed, and the fifth leaf had emerged to a distance of 30 to 110 mm. Previous dissections had shown that this degree of leaf development corresponds with lemma primordia at the early or middle stage of development of the hood "cushion," as previously described (STEBBINS and YAGIL 1966).

Photoperiodic treatments: Subjecting long day requiring Hooded-Atlas plants to short-day conditions resulted in a 7–9 week delay in flowering time and in the

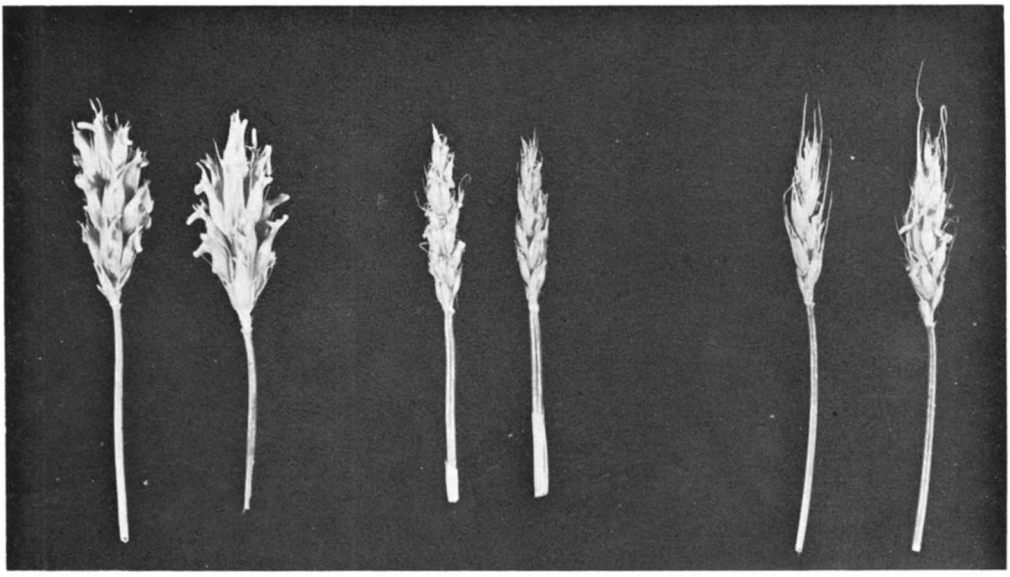

FIGURE 1.—Mature Atsel-Hooded spikes treated with a cold shock during the cushion stage of development (right), spikelet primordia stage (middle) and untreated control (left).

development of awned phenocopies similar to the ones obtained with the cold treatment. Figure 3 shows specimens grown under a short day compared to ones formed under a long day. The lemmas of the former are extensively elongated and the expression of the hooded structure is suppressed.

Several experiments were carried out in order to study the nature of this morphogenetic response to the photoperiodic treatments.

Table 1 shows the result of the four experiments. The first one consisted of three treatments: Plants grown in long day only (387 A-D), short day only (387 I-J) and some which grew 52 days under short-day conditions until reaching the spikelet primordia (SP) stage of development and were thereafter transferred to a long-day photoperiod (387 K-L). Only the ones treated throughout their entire growth period under short day produced phenocopies of awned. In the second experiment (3153 A-J), the short-day period followed by a long day, was extended to the beginning of lemma primordia (LP) development (3153 A-B) and to the stage where these primordia elongate (EL) (3153 C-D). Only in the second case, when plants were under short-day conditions during the stage of lemma elongation, did they respond significantly by developing awned phenocopies. Finally, two additional treatments were applied (112 A-H, 113 A-D) in order to determine whether this morphogenetic response to a short day is associated with the photoperiodic control of flowering or whether it is the result of some general retardation in growth due to the lack of photosynthesis or some other physiological process requiring light energy. In the first treatment (112 G,H) the plants were grown *first* under a long-day photoperiod until they reached the spike primordia (SP) stage of development and were thereafter transferred to a short-day regime. In

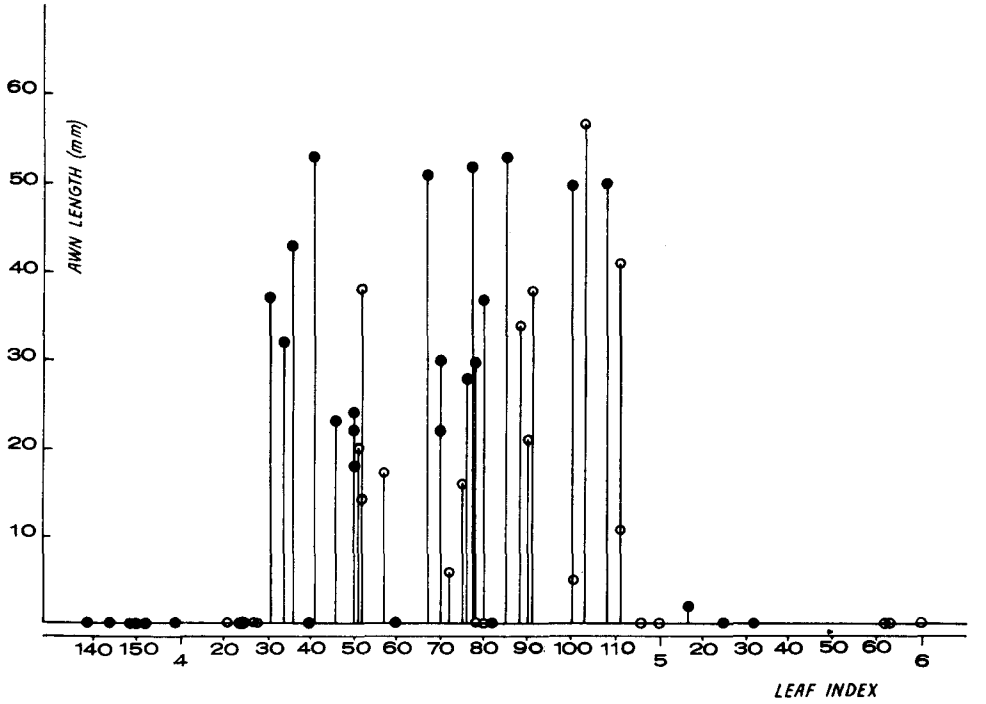

FIGURE 2.—Awn length of the longest awn produced in Atsel-Hooded genotype treated with cold shocks. Numbers on the abscissa represent stages of leaf development. The lower numbers indicate the number of leaves which have developed far enough so that their ligules are exposed; the upper numbers indicate the degree of emergence in mm of the next leaf. Black and open circles represent two experiments.

the second (113 A-D), the plants grew the entire period under a short day which was interrupted every night by 20 min of illumination with an incandescent lamp. As may be seen from the table, the differentiation of the hooded character was unaffected by either of these treatments. Furthermore, not all of the plants grown under short day up to the EL stage (112 C,D) responded as in the previous experiment which employed the same conditions (3153 C,D). In one of the replicates (112 D) the plants yielded awned phenocopies whereas in the other (112 C) most of them did not.

The phenotypic response of heterozygotes: Earlier workers have reported that the dominance of the hooded gene is incomplete (MURTY and JAIN 1960). Consequently, heterozygous plants were produced, and their phenotype as well as their response to a short-day regime were observed.

The heterozygotes were grown under three environmental regimes: (a) in a growth chamber under a long day; (b) in the greenhouse during the winter months under natural increasing daylength; (c) under a complete short day. The results are given in Table 2 and selected specimens are shown in Figure 4. They demonstrate that dominance is incomplete, since the plants grown under a com-

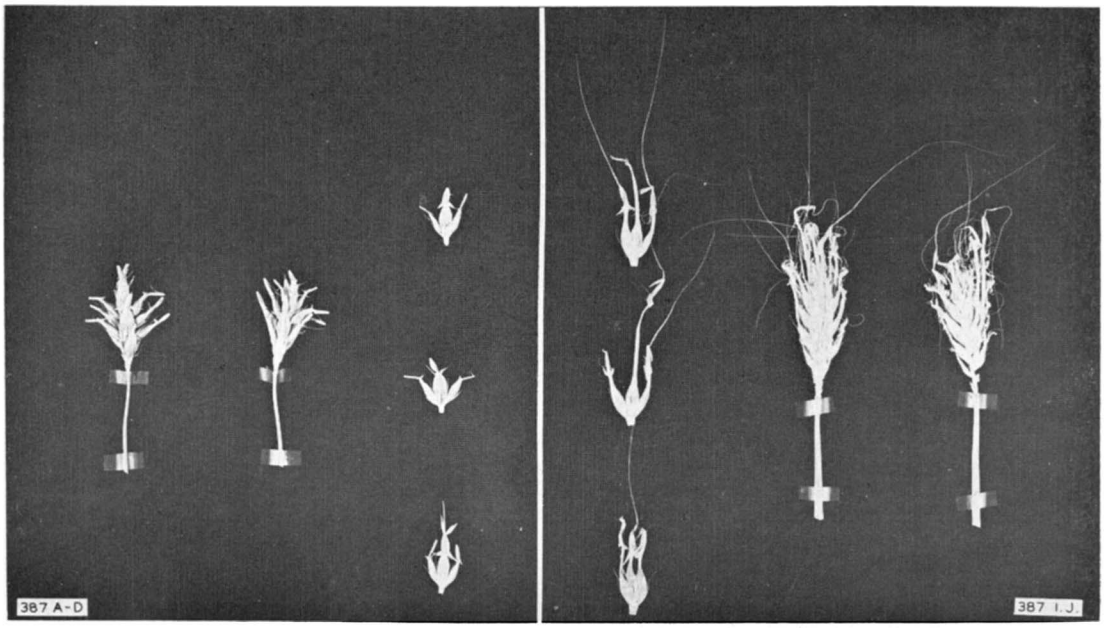

FIGURE 3.—On left—mature Atlas Hooded spikes grown under a 16 hr photoperiod. On right —mature Atlas Hooded spikes grown under an 8 hr photoperiod.

TABLE 1

Awn length of awned phenocopies in Hooded Atlas barley grown under various conditions

Pot No.	Days under short-day	Days under long-day	Days to flower	Length of longest awn (mm)	$\bar{X}$
387 A-D	..	44	44	all 0	0
387 I	107	..	107	42, 40, 51, 45, 42, 61, 59, 50, 64	50.4
387 J	107	..	107	45, 38, 48, 55, 43, 24, 64, 65, 64	49.6
387 K, L	52 (SP)	23	75	all 0	0
3153 I, J	..	58	58	all 0	0
3153 G	109–120	..	109–120	40, 22, 22, 13, 35, 21, 17, 38	26.0
3153 H	109–120	..	109–120	13, 17, 14, 38, 54, 51, 60	35.3
3153 A	60 (SP-LP)	17–24	77–84	0, 0, 0, 0, 0, 14, 4, 22, 19	5.1
3153 B	60 (SP-LP)	17–24	77–84	0, 0, 0, 0, 0, 0, 0, 0, 10, 32	4.2
3153 C	77 (EL)	18–30	95–107	54, 68, 24, 10, 0, 10, 32, 19, 22	29.9
3153 D	77 (EL)	18–30	95–107	62, 35, 45, 22, 47, 42, 37, 0, 37	36.3
112 E, F	..	56	56	all 0	0
112 A	124–130	..	124–130	67, 60, 56, 55, 29, 27, 26, 36, 41, 46	44.3
112 B	124–130	..	124–130	0, 67, 68, 57, 9, 54, 69, 47, 13, 47	43.1
112 C	76 (EL)	→ 19–27	95–103	0, 0, 0, 0, 0, 0, 17, 21	4.7
112 D	76 (EL)	→ 19–27	95–103	24, 14, 24, 10, 17, 15, 22, 22	18.5
112 G	22–25	← 46 (SP)	68–71	all 0	0
112 H	22–25	← 46 (SP)	68–71	all 0	0
113 A-D	(63) interrupted night	..	63	all 0	0

(SP)—Spikelet primordia, (LP)—Lemma primordia, (EL)—Elongating Lemma primordia.

TABLE 2

Awn length in heterozygous Hooded Atlas genotypes grown under various photoperiods

Pot No.	Treatment	Days to flower	Length of longest awn (mm)	$\bar{X}$
	Long-day in			
117 A, B	growth-chamber	70	0, 0, 0, 0, 26, 17, 24, 28, 14, 13	12.2
118 G	growth-chamber	70	0, 0, 0, 0, 10, 17, 17, 27	7.9
118 H	growth-chamber	70	0, 0, 0, 0, 0, 14, 26, 37, 49	12.6
	Long-day in			
117 D	greenhouse	80	44, 44, 4, 31, 14, 64, 12, 38, 0, 41	29.2
117 E	greenhouse	80	26, 0, 21, 52, 48, 72, 14, 28, 24, 30	31.5
118 I-J	greenhouse	82	70, 44, 44, 0, 28, 36, 48, 38, 28, 41	37.7
	Short-day in			
118 A	greenhouse	116–137	31, 12, 91, 21, 46, 41, 101, 51	49.2
118 B	greenhouse	116–137	99, 86, 66, 72, 67, 46, 41, 24, 59, 51	61.1
118 C	greenhouse	116–137	62, 122, 69, 65, 63, 95, 63, 52, 66	73.0
118 D	greenhouse	116–137	52, 33, 0, 110, 57, 54, 94, 36, 61, 19	51.6

plete long day (117 A,B; 118 G,H) showed a certain degree of lemma elevation with a considerable amount of variability. The degree of elevation increased with the delay of flowering and reached its maximum in the short-day treated plants (118 A-D), many of which yielded the best phenocopies produced by any of the treatments in the length of the awns as well as in the suppression of the hoodedness.

DNA synthesis: A measure of the frequency of nuclei synthesizing DNA in lemmas of both hooded and awned genotypes was obtained by the application of tritiated thymidine (^{3}H-T). The incorporation of the labelled nucleotide into DNA was observed by histo-autoradiography.

Labelled nuclei were obtained in all treated lemmas. The percentages of labelled nuclei in the adaxial epidermal cells in the inner tissue and the total percent in the region of the differentiation were plotted as a function of anther length (see STEBBINS and YAGIL 1966), and the result is presented in Figure 5. Despite the variability of the data the increase in percent of labelled nuclei in hooded lemmas is evident. This increase commences somewhat in advance to cushion differentiation, i.e. during the terminal period of the Elongating Lemma Primordia (EL) stage, and reaches a maximum when the cushion commences to be visible. At that time the percent of labelled nuclei in the hooded lemmas is approximately 3× higher than in the awned. Due to the high variability of the data no significant differences were found between epidermal cells and those of the inner tissues. Longitudinal sections of treated lemma primordia from hooded and awned genotypes have been illustrated elsewhere (STEBBINS 1965, Figure 5; 1967, Figure 8).

RNA synthesis: RNA synthesis was studied by autoradiography with tritiated cytidine (^{3}H-C). The label appears as black dots over the cytoplasm and nuclei (Figure 6 A,B). As with DNA, a higher rate of RNA synthesis associated with the differentiation of the hooded cushion is evident. The number of grains per unit

FIGURE 4.—Heterozygous Hooded Atlas grown under various photoperiods. See Table 4 and text for explanation.

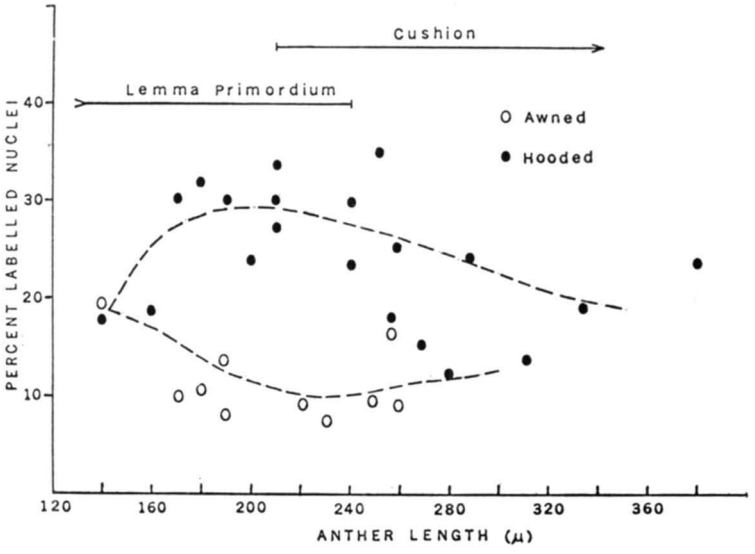

FIGURE 5.—Percent of ^{3}H-T labelled nuclei in median longitudinal sections of lemma primordia. Open circles-awned genotype. Black circles-hooded genotype.

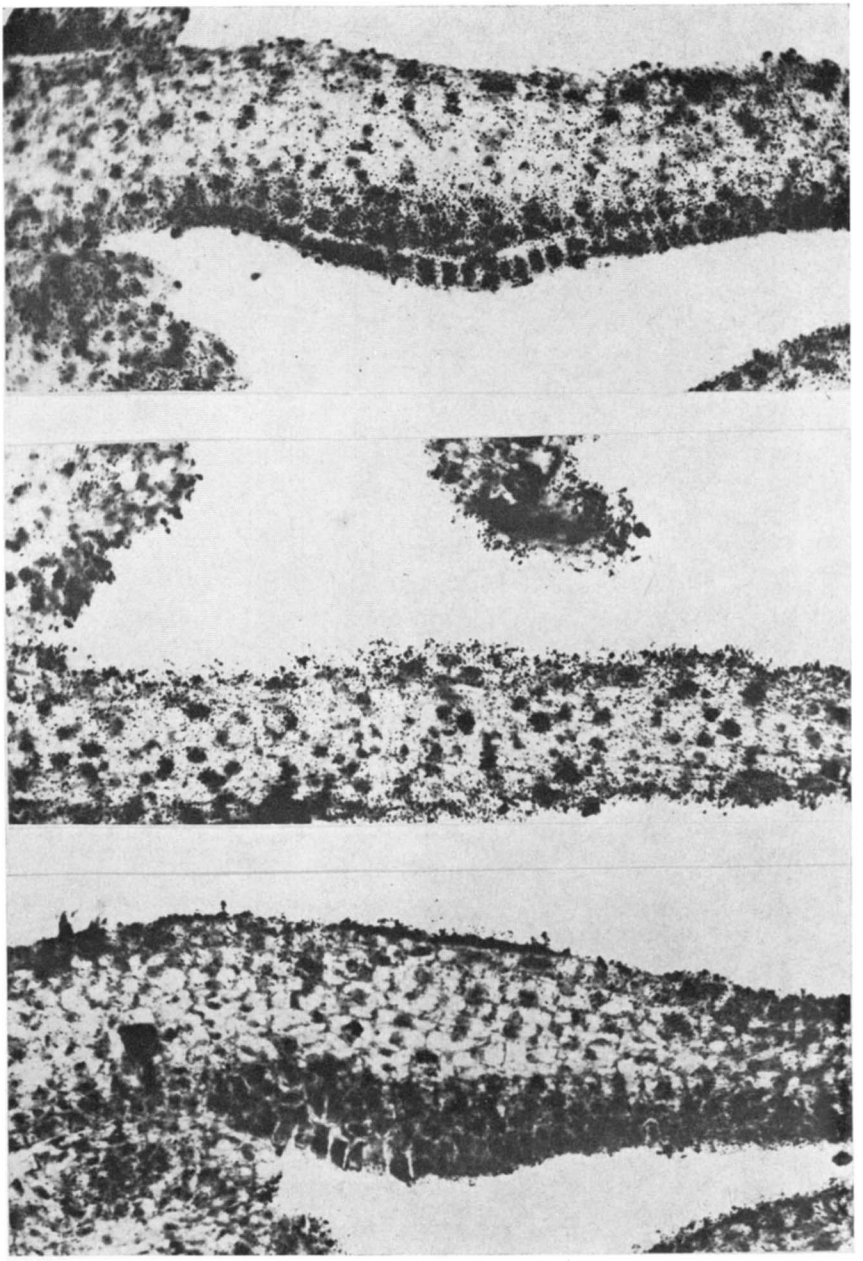

FIGURE 6.—A, B,—autoradiographs of median longitudinal sections of lemma primordia treated with ^{3}H-C. A—hooded genotype at cushion stage. B—awned genotype at EL stage. C—median longitudinal section of a hooded lemma at the cushion stage stained with pyronin–methyl green. (×300).

area over the adaxial epidermis and 2–3 cellular layers beneath it was determined. In Figure 7 the results are plotted against anther length. The density of the label remains almost unchanged in awned lemmas whereas in the hooded ones there is a shift upwards at the end of EL stage. At the beginning of the cushion stage it reaches a plateau. Figure 6 C shows a hooded lemma at early cushion stage stained with pyronin methyl-green. The cytoplasm of the cushion cells is heavily stained with pyronin (which labels RNA, see JENSEN 1962). This again, demonstrates the increased concentration of RNA in association with cushion differentiation.

DISCUSSION

Environmental factors: The experimental results reported have demonstrated that two basic environmental factors, temperature and light, alter the phenotypic expression of the hooded genotype. Under either extreme temperature or abnormal daylength, both of which slowed down considerably the rate of development, the hooded gene partially or completely failed to express its normal phenotype and tended to produce awned phenocopies.

Retardation of cell division rate due to low temperatures has been demonstrated in root meristems (BROWN and RICKLESS 1949; BAILEY 1954) and in the alga Chlorella (TAMIYA 1963). One can reasonably postulate, therefore, that in our example, the cold treatment given in the hooded seedlings slowed down cell division without affecting cell elongation. Thus, given at the right time, the cold treatment prevented the enhanced frequency of mitoses which initiates development of the hooded cushion (STEBBINS and YAGIL 1966). As a result, a slower division cycle, followed by a period of elongation, which usually takes place in the awned genotype, took place in primordia of the hooded genotype, thereby producing the one dimensional development of an awn.

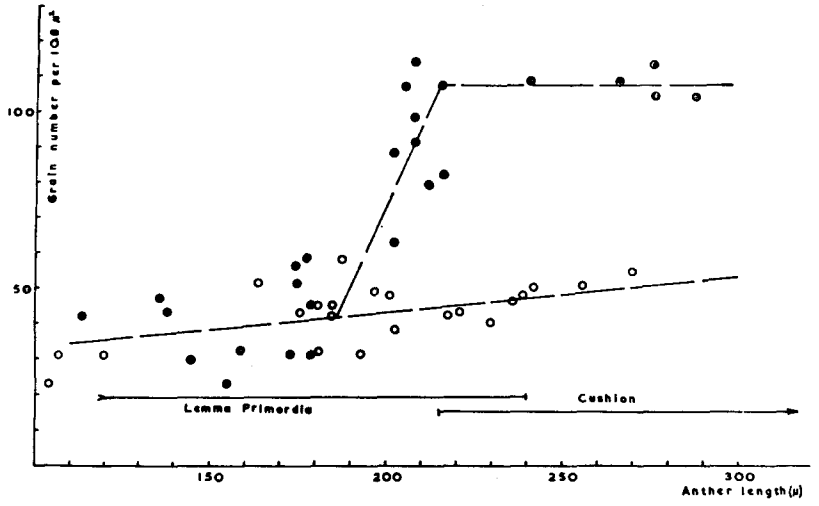

FIGURE 7.—Grain number in adaxial side in ^{3}H-C autoradiographs of developing lemmas of the awned (open circles) and hooded (black circles) genotypes.

The effect of the short-day treatment on the production of awned phenocopies was to retard greatly the development of the reproductive apical meristem. Since this meristem is characterized by a high frequency of mitosis under normal conditions, the retardation of its development might be expected to shift the cell division-elongation balance toward greater elongation. The fact that floral primordia are formed in a long-day barley variety under short-day conditions has also been reported by ASPINALL and PALEG (1963) and PALEG and ASPINALL (1964) who further reported a slower development of the apical meristem under short day as well as low light intensities. EVANS (1960) showed in Lolium that long-day induction quantitatively speeded up the development of the reproductive shoot apex. In the hooded case, it required twice the time for the short-day treated plants to flower. Although no quantitative data are presented, observations indicated that each stage of apical differentiation lasted much longer under short-day conditions.

The phenotypic response of the hooded plants in both treatments, cold shocks as well as short-day regime, was restricted to a critical period of lemma differentiation, i.e. at the time when the hooded cushion was beginning to form. The shortness of this critical stage of gene action can be seen from Table 7 where only one group of plants in the EL stage (112 D) reached it at the time of treatment, whereas the other (112 C) was transferred to a long-day regime before attaining this stage. In this example, the stage of development was determined approximately by dissecting some of the plants and treating others which resembled them in the stage of leaf development. The importance of the time of treatment in the production of phenocopies has been demonstrated in various animals as Drosophila, Ephestia, chicken and mice (GOLDSCHMIDT 1955; HADORN 1961; LANDAUER 1958). As also argued by these authors, the nature of the treatment and the most effective period of its action during development reflects the nature and time of the action of the hooded gene on the primary meristem.

We have postulated previously (STEBBINS and YAGIL 1966) that the hooded gene initiates a new epigenetic sequence of reproductive organ differentiation. We believe that this sequence is initiated by an accelerated mitotic rhythm resulting in the organization of a three dimensional structure—the cushion—which has the properties of a floret primordium. The fact that the alteration of a single physiological factor (temperature or daylength) at the time of this initiation can alter or even cancel all morphological differences between the two genotypes suggests that a single effect of the hooded gene is an alteration of the mitotic rhythm and that all the remaining stages of hood differentiation take place as a consequence of this alteration. Furthermore, our observation that phenocopies are easier to obtain when one gene dosage is present (in heterozygous plants) than when two are present (in homozygotes) further supports the idea that the change of mitotic rhythm giving rise to the hooded cushion is the result of the action of the hooded gene. Since this gene action may very likely be via a change in hormone constitution in the distal end of the lemma primordia (STEBBINS and YAGIL 1966), it is also likely that the phenocopies reported here were produced by the action of cold or short-day treatment in altering the hormonal balance.

The fact that the interrupted night treatment cancelled the short-day effect supports this hypothesis.

An alternative hypothesis can be suggested on the basis of experiments now being conducted in our laboratory. The hooded genotype has been found to possess at most stages of development a much higher activity of peroxidase than awned. This activity reaches its maximum at a stage just preceding the one at which a histological difference between the two genotypes can be detected, and which is sensitive to phenocopy modification by the action of cold or short days (GUPTA and STEBBINS 1969). Furthermore, inhibitors of peroxidase activity, such as sodium azide, when applied to apical meristems of hooded at this stage, produce phenocopies similar to those reported in this paper (STEBBINS and GUPTA in press). Consequently, the direct action of cold and short days may be upon peroxidase activity.

DNA synthesis: In a previous communication (STEBBINS and YAGIL 1966), the writers have shown that the differentiation of the cushion of tissue which in the hooded genotype will give rise to extra floral parts is induced by the following events: Gradual decrease in mean cell length, due to a smaller amount of cell enlargement during the interphase between mitotic divisions; increase in the frequency of mitoses; change in the orientation of mitotic spindles from one plane to three. On the basis of these observations we suggested that the hooded gene initiates a new epigenetic sequence of reproductive organ differentiation, triggered off primarily by the increase in mitotic frequency.

The results of the experiments with radioactive tracers suggest that the increased frequency of mitoses, which by reducing the length of interphase automatically brings about a reduced amount of cell enlargement between successive mitoses, is closely associated with and perhaps caused by changes in the timing of DNA synthesis. According to present concepts, the interphase can be divided into three periods: G_1, the period of growth before DNA replication; S, the period of actual replication; and G_2, the period between the completion of replication and the onset of mitotic prophase. Our data indicate that the shortening of interphase cannot be due to a shortening of the S period, i.e. a more rapid synthesis of DNA. If this were so, the proportional time spent by the interphase nucleus in DNA synthesis would not differ in the two genotypes, and the great increase in the frequency of nuclei synthesizing DNA at any one time would not have been observed. Unless mitotic divisions are synchronized, which is impossible in our material, one cannot obtain positive evidence as to whether the shortening of an interphase period is due to reduction in length of G_1 or G_2. Our reasons for believing that the G_1 period is chiefly involved are based upon the observations by one of us (STEBBINS 1965) on the combination of mitoses and differentiation processes which take place in the epidermis of barley leaves. In these sequences, the timing of successive mitoses can be followed by observing the relationship of an interphase cell to the surrounding nuclei and cells. In the differentiating cells of this tissue, DNA synthesis takes place shortly before the onset of mitotic prophase; i.e. the G_2 period is relatively short compared to G_1. Regulation of mitotic frequency appears to be accomplished by changing the timing of the onset of DNA

synthesis after the completion of the previous mitosis. It is a function of the length of the G_1 period. By analogy, it seems to us most probable that the same kind of regulation is operating in the lemma primordia.

The relationship of this regulation of the timing of interphase to the primary action of the hooded gene is by no means clear. Attempts to study it in isolated shoot apices have failed, since no combination of nutrient materials or growth substances has yet been found which will promote the growth of embryonic reproductive shoots *in vitro*. Since cultures which included stem leaves attached to the reproductive apex have given rise to fully mature leaves without any development of the reproductive spike, this failure to develop *in vitro* is apparently specific to the embryonic shoot itself. Apparently it requires substances which are synthesized in another part of the plant, either the roots or the more mature tissue of the crown.

SUMMARY

Hooded barley plants were subjected to either cold treatment or a short-day photoperiod. As a result many of the lemmas elongated and formed awned phenocopies. The treatment was effective only when given during the developmental period when the hooded gene begins to produce a visible effect on the histological development of the primordium. In every case, the formation of awned phenocopies was associated with a retardation of development. Short-day treatments, even those applied at the critical developmental stage, which did not retard development, failed to produce awned phenocopies. These results support the authors' hypothesis that all of the morphological effects of the hooded gene result from the alteration at a critical stage of a single process, the frequency of cell division relative to cellular elongation. This hypothesis is supported by experiments using autoradiography with precursors of DNA and RNA synthesis. An increased frequency of nuclei synthesizing DNA begins before a difference in the frequency of mitoses can be observed, suggesting that the morphogenetically significant effect of the hooded gene is to shorten the G_1 period, between mitotic telophase and DNA replication, in nuclei located on the adaxial surface of lemma primordia which are 150–200 micra long.

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