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Effect of Light and Gibberellin on Ribonucleic Acid Species of Pea Stem Tissues as Studied by Deoxyribonucleic Acid-Ribonucleic Acid Hybridization[1]

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ABSTRACT

The ability of gibberellin and light to alter gene transcription in dwarf pea (*Pisum sativum* L., var. Progress No. 9) stem issues has been investigated by means of DNA/RNA hybridization-competition techniques. Distinct changes in hybridizable RNA are caused by a 24-hour pretreatment of the seedlings with light, but no changes in RNA were detected up to 50 hours after treatment of the seedlings with gibberellin. Gibberellin is similar to auxin in its ability to induce stem growth without causing detectable changes in hybridizable RNA.

The ability of gibberellins to regulate protein synthesis in higher plants has been clearly demonstrated in the case of several hydrolytic enzymes in barley aleurone layers, but less is known concerning the effects of these hormones on RNA synthesis (12). Studies with inhibitors have suggested that RNA synthesis is essential for the realization of ultimate physiological responses to GA in a variety of systems, but these experiments do not differentiate between a simple requirement for continued production of the same RNA species and an actual requirement for transcriptional changes resulting in synthesis of new species of RNA. Since inhibitors of RNA synthesis would be expected to block all subsequent processes requiring a continued supply of new RNA, control by GA could equally well be exerted at some step subsequent to transcription. Experiments have already been reported which support such a possibility in the case of GA-induced induction of germination in wheat embryos (3) and invertase synthesis in sugar cane (6).

Possible effects of GA on transcription *per se* have previously been investigated by determining rates of RNA synthesis in isolated nuclei or chromatin preparation (9–11, 14). This approach is necessarily indirect, however, and does not permit direct comparisons of base sequences. Furthermore, seemingly contradictory results have been obtained. For example, Johri and Varner (11) reported that nuclei isolated from dwarf peas in the presence of GA showed enhanced RNA synthesis and that the newly synthesized RNA differed in size distribution and nearest neighbor frequency from that synthesized in control nuclei. But McComb *et al.* (14), using chromatin from internodes of seedlings previously treated with GA, failed to find any increase in total template capacity measured in the presence of added *E. coli* RNA polymerase. Thus it remains uncertain whether GA changes the capacity of pea chromatin for RNA synthesis.

Increases in total template capacity of chromatin have been reported in other systems in response to GA treatment (9, 10), but it is uncertain that these changes in ability to support *in vitro* RNA synthesis mean that similar changes occur *in vivo*. This distinction is especially important in view of the existence of multiple RNA polymerases in higher organisms (17) and the dramatic effects of subunit structure on template specificity of bacterial enzymes (2). Much further work is necessary before it will be possible to make unambiguous statements concerning *in vivo* transcription based on *in vitro* experiments.

The most direct approach to the question of whether or not transcriptional changes are involved in a developmental response requires comparison of RNA base sequences present *in vivo*. Currently, this is possible only with DNA/RNA hybridization techniques. In the work to be presented, hybridization experiments have been conducted in order to provide a direct comparison of certain of the RNA base sequences present in dwarf pea seedlings treated with or without GA_3. In addition, the effect of development in the light as opposed to darkness has been examined by means of hybridization, and results for the two responses have been compared.

MATERIALS AND METHODS

Seeds of dwarf peas (*Pisum sativum* L., var. Progress No. 9) were germinated in moist vermiculite and grown in the dark (with occasional dim green light) for 6 to 7 days at 25–28 C. Batches of seedlings were then transferred to a growth chamber with approximately 1000 ft-c of fluorescent and incandescent illumination at 25 C for experimental treatment. This procedure, which is modified from that of Phillips and Jones (16), results in plants of a more convenient size for experimental manipulation than seedlings grown continuously in the light. In addition, it allowed us to compare GA and light treatments simultaneously.

GA treatment consisted of spraying seedlings to runoff with a solution of 0.01% GA_3 (Calbiochem, K salt) in 50% ethanol containing 0.05% Tween-80. Control plants, both in the light and in the dark, were treated at the same time with the same solution without GA. At various times after treatment, the

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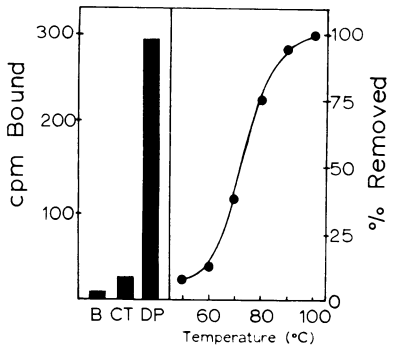

FIG. 1. Left, comparison of the ability of RNA to hybridize with homologous and heterologous DNA. ^{32}P-labeled dwarf pea RNA was incubated for 22 hr at 38 C with dwarf pea (DP), calf thymus (CT), or blank (B) DNA filters. Calf DNA was obtained commercially (Worthington) and purified by phenol extraction and precipitation with cetyltrimethylammonium bromide (19). Right, thermal dissociation profile of hybrids formed with dwarf pea DNA in the preceding experiment. Hybrids were dissociated in 0.15 M NaCl-15 mM tris-HCl, pH 7.0. At each temperature, filters were washed twice, the eluates combined and counted in 15 ml Aquasol scintillation fluid (New England Nuclear). Radioactivity removed is expressed as a cumulative percentage of the total removed at all temperatures.

epicotyls were excised and frozen in Dry Ice prior to RNA extraction.

Isotopic labeling of RNA was accomplished in the light by placing 5 to 10 excised epicotyls with their bases in small vials containing 200 μc of carrier-free $H_3{}^{32}PO_4$ (New England Nuclear) in 0.5 to 2.0 ml of medium containing 50 μM chloramphenicol, 100 μM penicillin G, and 2.5 mM potassium maleate buffer, pH 4.7. A readily observable growth response to GA was obtained with epicotyls treated under these conditions (16, and unpublished observations).

Preparation of nucleic acids, immobilization of DNA on nitrocellulose membrane filters, and hybridization techniques were as described previously (19). Experiments were conducted essentially as described by Gillespie and Spiegelman (7) except for the use of formamide to permit reactions to be run at lower temperatures (15). All experiments were conducted in 0.3 M NaCl–30 mM sodium citrate containing 40% formamide and 0.1% sodium dodecyl sulfate. Specificity of the hybridization reaction was monitored by determining the degree of cross reaction between unrelated nucleic acids and by thermal dissociation experiments (Fig. 1). The degree of specificity achieved, though not absolute, is comparable to that commonly achieved in experiments with mammalian nucleic acids and is the same as that reported previously for Alaska pea nucleic acids (19).

RESULTS AND DISCUSSION

In a standard hybridization-competition experiment, different preparations of unlabeled RNA are tested for their ability to compete for DNA sites against a radioactively labeled reference RNA preparation. This is accomplished by adding increasing concentrations of each unlabeled RNA to a series of vials containing a fixed amount of filter-bound DNA and labeled RNA. The degree of identity between labeled and unlabeled RNA species capable of hybridization is judged by the degree to which the unlabeled RNA reduces the hybridization of labeled RNA. Thus, if a given preparation fails to contain certain of the RNA species present in the reference population, it will produce a competition curve which reaches a plateau somewhere above the corresponding curve for a fully homologous preparation of unlabeled RNA.

Figure 2 shows the results of such an experiment using unlabeled RNA from dark-grown plants or plants exposed to light for 24 hr prior to treatment with or without GA for a further 6 hr in the light. Reference RNA was obtained from seedlings treated with light and GA as described above and labeled with ^{32}P throughout the 6-hr period of GA treatment. It can be seen that RNA preparations from plants treated in the presence or absence of GA produce indistinguishable competition curves. Therefore, no new species of RNA which can be detected by this technique are synthesized in response to GA during the first 6 hr after treatment. In contrast, RNA from dark-grown seedlings fails to compete fully with RNA from light-treated plants. Thus, RNA synthesized between 24 and 30 hr after transfer to light includes species which are not detectable in dark-grown seedlings, and whose synthesis may therefore be said to be induced by light treatment. It is unlikely that the entire light effect can be attributed to RNA synthesis in the chloroplasts, since chloroplast DNA constitutes a very small percentage of the total DNA, and RNA hybridized to chloroplast DNA would not be expected to account for a significant fraction of the total hybrid formed.

Detection of light-induced RNA species under conditions which fail to reveal any effect of GA treatment is an important internal control in these experiments, since it shows that the assay is indeed capable of discriminating between different populations of nucleic acids from the same plant tissues. One must conclude, therefore, that any changes in the pattern of RNA synthesis induced by GA during the first 6 hr after its application must either involve only a small fraction of the total hybridized RNA or be confined to those species of RNA which fail to hybridize under our conditions; any changes induced by GA in the pattern of transcription must be quite different from those induced by light.

If synthesis of significant quantities of hybridizable RNA species were required prior to initiation of GA-induced growth, these species should have been detectable in the preceding ex-

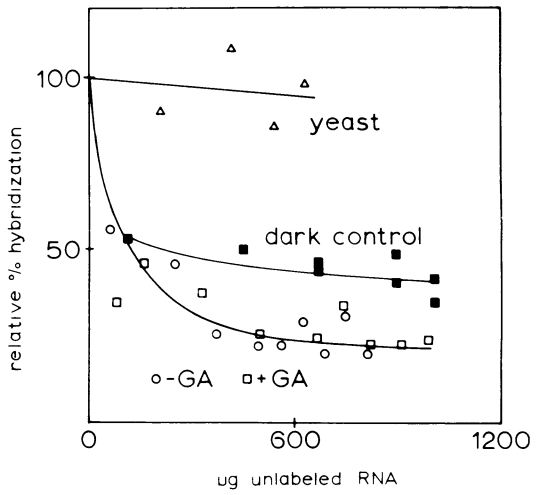

FIG. 2. Comparison of the effect of light and GA on hybridizable RNA. RNA was harvested from plants grown 30 hr in the light ± GA during the last 6 hr. RNA for dark controls was harvested from dark-grown plants of the same age. Reference RNA was obtained by labeling light-grown plants during the GA treatment. Indicated amounts of each unlabeled RNA were mixed with 17 μg ^{32}P reference RNA from plants treated with both GA and light and incubated in 0.2 ml with 17 μg filter-bound DNA for 45 hr at 45 C.

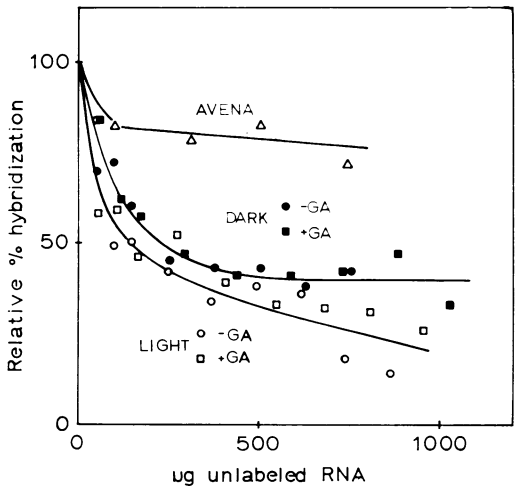

FIG. 3. Absence of changes in hybridizable RNA following 32 hr of GA treatment. Plants were treated with or without GA in light or darkness as described in the text, and ^{32}P reference RNA was obtained from plants treated with both GA and light. Indicated amounts of unlabeled RNA were combined with 50 μg reference RNA in 0.175 ml and allowed to react with 17 μg filter-bound DNA for 36 hr at 40 C. Specific radioactivity of the reference RNA was 806 cpm/μg and 100% hybridization was 212 cpm/filter.

periment, because the 6-hr treatment period employed is approximately the same as the lag period before GA-induced growth is detectable in dwarf pea internodes (14). However, it is also possible that new species of RNA could be induced later, after GA-induced growth is in progress. This could occur either as a delayed response to the hormone itself or as a secondary consequence of hormone-induced differentiation events. Several experiments were therefore conducted in which RNA was collected at different times after treatment with GA. In one such experiment, seedlings were treated for 32 hr in the light or in darkness, with or without GA, and the RNAs obtained were compared with reference RNA from plants treated with GA in light and labeled between 24 and 32 hr. As shown in Figure 3, it was impossible to detect the presence of GA-induced species at this time. Similar experiments conducted after 12 and 50 hr of GA treatment also failed to reveal any changes in hybridizable RNA species resulting from GA treatment. Transcriptional events comparable to those induced by light therefore do not occur in response to GA either before or during the period of GA-induced growth.

Complete absence of transcriptional changes associated with GA action cannot be inferred from the data presented here, however. The present experiments have been designed to detect only the induction of new RNA species, and hormone-induced repression of RNA synthesis would not have been observed. Furthermore, GA-induced synthesis of RNA comprising only a small fraction of the total hybridizable radioactivity would not have been detected.

In addition, competition experiments with nucleic acids of higher organisms generally measure only the minimum difference between two RNA preparations and cannot be used to prove that any two preparations contain exclusively identical base sequences. For example, changes may occur in the synthesis of RNA complementary to the unique fraction of the genome, which would fail to hybridize under our conditions. DNA reassociation experiments reported by Sivolap and Bonner (18) indicate that about 60% of the pea genome consists of sequences exhibiting various degrees of repetition. Thus, even if it is assumed that this entire fraction is subject to hybridization, 40% of the DNA will not be measured in the present experiments. It is also possible for transcriptional changes within the partially redundant fraction of the genome to be obscured if the sequences involved are similar enough to cross-react with other sequences under the conditions used. These problems have been more fully discussed elsewhere (13, 19).

In spite of these difficulties, certain inferences regarding GA action may still be drawn from these experiments. Britten and Davidson (1) have proposed that nuclear RNA species complementary to the partially redundant fraction of the genome may be involved in coordinating transcriptional changes. Such molecules would be expected to hybridize under most conditions, and the absence of detectable changes in the present experiments indicates that any transcriptional changes which may occur in response to GA do not involve substantial changes in these postulated regulatory RNA species. In addition, at least a portion of the mRNA from HeLa cells has been shown to hybridize under conditions similar to ours (5), and various hormonal responses in animal systems have been shown to involve readily detectable changes in hybridizable RNA. In plants, similar changes may be detected in Alaska pea stem sections in response to high levels of 2,4-D (19), and the present work has shown changes in response to light in the same tissues used for GA experiments. It is not necessary to assume that all these changes involve many different genes (or RNA species), since RNA even from a single gene would be detectable if it comprised a large enough fraction of the total radioactivity hybridized. Thus it is clear that the GA response is different from all the above, since if transcriptional changes occur at all they must be less extensive or involve a different portion of the genome. This situation is similar to that of promotion of stem elongation by physiological levels of auxin, a process which also does not appear to involve changes in hybridizable RNA (19).

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