Rapid Initiation of Thymidine Incorporation into Deoxyribonucleic Acid in Vegetative Tobacco Stem Segments Treated with Indole-3-acetic Acid

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ABSTRACT

The short term effect of 11.4 μM indoleacetic acid on the incorporation of (methyl-3H)thymidine into DNA in vegetative tobacco (Nicotiana tabacum cv. Wis. 38) stem segments has been investigated. In segments that are defoliated, inverted, and kept in the dark for 7 hours, indoleacetic acid very rapidly (about 60 minutes) and strikingly initiates thymidine incorporation into DNA. The time required before enough indoleacetic acid (2.8 μM) to enhance thymidine incorporation moves into a segment has been found to be about 35 minutes. The initiation response time for segment tissue that already contains 2.8 μM indoleacetic acid should be no more than about 25 minutes. The rate of labeled thymidine incorporation into DNA is affected by physiological treatments of segments. Moving segments from the light into the dark or defoliating segments or inverting defoliated segments decreases the rate of thymidine incorporation. For segments given all three treatments, indoleacetic acid restores the rate of thymidine incorporation as compared to controls. Darkness, or defoliation or inversion of segments, therefore, may decrease thymidine incorporation into DNA by effecting reduced auxin levels in stem segments.

Tissue excised from plants that are already flowering possesses the capacity to initiate flower buds de novo when cultured in vitro (2, 6, 14, 18, 21, 22, 27, 28, 30). In a detailed study of this phenomenon using tissue cultures of tobacco, Aghion-Prat (1, 2) discovered the existence of an inhibition gradient in the flowering capacity of tissue excised along the stem axis of a flowering plant. The inhibition gradient of flower bud formation described by Aghion-Prat has been shown to occur simultaneously with a promotion gradient of vegetative bud formation (30). The inhibition floral gradient is actually a transition to vegetative bud development rather than an inhibition of budding. Aghion-Prat (2) has suggested that the floral gradient in the stem of a flowering tobacco plant may be due to gradients of endogenous growth substances (unspecified). In support of this idea, Wardell and Skoog (31) have found that exogenous application of IAA to stem tissue excised from the top (above the gradient) of a flowering plant and cultured in vitro resulted in a transition in the tissue to vegetative bud formation strikingly similar to the gradient itself. RNA base analogs, which were shown to completely counteract the action of IAA on bud expression, effected in stem tissue below the gradient a transition to flower bud formation comparable to tissue above the gradient and not treated with base analogs. On these bases, Wardell and Skoog (31) have proposed that the gradient in bud expression may be due to an inverse auxin gradient in the stem. Wardell (29) has suggested the possibility that an inverse auxin gradient could result from auxin in young leaves being transported down the stem, thus increasing the concentration of auxin in the direction of the base. Consistent with this idea, young leaves along the stem of a tobacco (Nicotiana tabacum cv. Purpurea or cv. Connecticut Broadleaf) plant have been shown (4) to be high in transportable auxin.

Recently, a correlation between in vitro floral expression and DNA content in stem segments from a flowering tobacco plant has been reported (32). The DNA content in stem segments above the gradient was 10-fold greater (on a fresh weight basis) than in segments below it, and the DNA content decreased roughly in parallel with the gradient itself. Low concentrations of IAA, effective in inhibiting floral expression in vitro (30), also inhibited (H)thymidine incorporation into DNA; but in comparative experiments using stem segments that are known to form only vegetative buds in vitro (i.e., segments from a vegetative tobacco plant), the same low IAA concentrations promoted (H)thymidine incorporation into DNA. In other words, low concentrations of IAA have opposite effects on incorporation of thymidine into DNA in floral-induced and vegetative tobacco stem segments.

Taken together, the studies by Wardell and Skoog suggest that the effects of IAA on thymidine incorporation into DNA in stem segments from flowering and vegetative tobacco plants may be related to the effects of IAA on bud expression of cuticular stem segments. Possibly, IAA effects reversible qualitative change in total DNA in favor of vegetative bud formation in floral-induced segments, and IAA maintains total DNA in favor of vegetative bud formation in vegetative segments. On the basis of this possibility, further work has focused on DNA. The experiments reported here attempt primarily to establish how quickly IAA effects an increase in thymidine incorporation into DNA in stem segments excised from a vegetative tobacco plant.

MATERIALS AND METHODS

Growth of Plants and Labeling of Stem Segments. Vegetative tobacco (Nicotiana tabacum cv. Wis. 38) seedlings were...
transplanted from an 18-cm saucer into 5-cm Jiffey pots and were grown under cheesecloth in the greenhouse until they were about 15 cm tall. The shoots were harvested; all leaves below the two uppermost expanded ones were removed; and 3 cm of the stem bottoms were cut off. For each treatment (10 experiments consisted of about 10 treatments), the stem bottoms of two shoots were placed in contact with 0.25 ml of labeling solution (0.1 mM (methyl-3H)thymidine, 25 μCi/ml chloramphenicol) containing a 5-ml beaker. Then the shoots were illuminated as previously described (32). They absorbed the labeling solution in 30 min. Fifteen min later (total uptake period of 45 min) they were removed from the beaker, and usually they were decapitated. The resulting defoliated stem segments (9.5 cm) were placed, depending upon the experiment, either upright or inverted into 4 ml of test solution contained in a 10-ml beaker. Then, the treatments were incubated at room temperature in the dark for time intervals specified in the figures. Following incubation, the segments were quickly frozen, by placing them on dry ice and were stored in a freezer.

Isolation of DNA. For each treatment, the tops (1 cm) and bottoms (1 cm) of the two segments were cut off. Of the remaining stem tissue (7.5 cm), a fraction containing the DNA was extracted from a homogenate as previously described (32) but with the following changes. One mg of unlabeled spooly fraction (1.25 mg/ml) extracted from stem tissue homogenates of flowering tobacco plants (32), was added as carrier to 5 ml of grinding medium contained in a mortar. (Warrell and Skoog have reported that DNA cannot be isolated from vegetative stem tissue homogenates alone.) After grinding the tissue, the macerate was transferred into a 15-ml corex centrifuge tube. Any macerate remaining in the mortar was washed out with 2 ml of grinding medium and also was transferred to the centrifuge tube. The combined macerate (about 7 ml) was immediately centrifuged (unless otherwise specified, centrifugations were at 12,000 g for 10 min at 4°C). To precipitate the nucleic acids, ethanol was mixed with the supernatant, and the mixture was stored overnight at −10 °C. To remove rRNA, the ethanol precipitate was extracted with 3 M NaCl (2 ml), centrifuged, reextracted (1 ml), and centrifuged. The supernatants were combined and again were centrifuged (17,000 g for 10 min at 4°C). The supernatant was mixed with ethanol. A precipitate formed which, following centrifugation, was rinsed in rinsing solution (80% ethanol, 0.1 mM sodium acetate, pH 7). After centrifugation, the pellet was dissolved in 2 ml of buffer solution (0.14 M NaCl, 20 mM tris-Cl, pH 7.3). This solution is referred to as the high salt soluble fraction.

The foregoing procedure was repeated for each of the different treatments/experiment. Then the nucleic acid concentrations (0.5–0.6 mg/ml) in the separate high salt soluble fractions were determined from absorbancies at 260 nm (1.0 A260 unit = 50 μg of nucleic acids) of 0.05-ml samples (2 replicates/sample) each diluted with 0.01 ml 1 M MgCl2 and 0.94 ml H2O. Next, the radioactivities in the samples were measured as described previously (32) except that a Nuclear-Chicago Mark 1 liquid scintillation system was used for counting. The remaining portions (1.9 ml/treatment) of the separate fractions per experiment were pooled, and the concentration and the radioactivity of the pooled fraction were determined (duplicate 0.05-ml samples). The rest of the pooled fraction was mixed with solid sodium perchlorate, and the DNA was extracted (32) without any further changes. Then, the absorbancy of the DNA and the radioactivity in the DNA were measured. A portion of the DNA was mixed with marked tobacco (32)DNA, and the mixture was analyzed by isopycnic CsCl density gradient centrifugation (8).

Transport of IAA. Four 5-cm segments were inverted, and their stem tops were placed into 1 ml of 2-3H)IAA solution (50 μCi) contained in a single 10-ml beaker. Thirty minutes later, the inverted segments were transferred to separate 10-ml beakers, each containing 4 ml of unlabeled IAA solutions (11.4 μM). Then the segments and beakers were placed in the dark at room temperature for different periods of time (i.e., 30, 60, 90, and 120 min). After the time period had elapsed for a segment, 1 cm of both ends were cut off, and the remaining stem segment (3 cm) was sliced immediately into 30, 1-mm sections. Next, each section was homogenized separately in a tissue homogenizer (Ten Broeck, 7 ml working capacity) containing 0.5 ml of buffer solution. The section homogenate was transferred into a 20-ml scintillation vial, and the homogenate remaining in the homogenizer was transferred to the vial by using an additional 0.5 ml of buffer solution. After homogenizing all the sections of a segment, 2 ml of Bio-Solv plus 10 ml of scintillation fluid (32) were added to each vial, the vials were vortexed, and the radioactivities were measured.

Estimated 2-3H)IAA CPM/Segment Section Equivalent to 2.8 μM IAA. The fresh weight volume of a cylindrical (0.1 cm x 0.6 cm) segment section was calculated (0.028 cc). And the dry weight/fresh weight ratio of a segment was determined (0.1). On these bases, the aqueous section volume was estimated to be about 90% (0.025 cc) of the total section volume. Since the specific radioactivity of the 2-3H)IAA (formula weight: 195) was 55 mCi/mmole and since the radioactivity in the IAA labeling solution (50 μCi/ml) was experimentally determined to be 78 x 10^5 cpm, a 0.025 cc aqueous section volume containing 2.8 μM 2-3H)IAA was equivalent to about 60,000 cpm.

Source of Chemicals. Bio-Solv was purchased from Beckman Instruments, Inc. (Methyl-3H)thymidine (20 Ci/mmole) was a product of New England Nuclear Corp., and the ammonium salt of 2-3H)IAA (55 mCi/mmole) was a product of Amer sham/Searle. Crystallized RNase and DNase, electrophoretically pure, and pronase were from the Worthington Biochemical Co. Chloramphenicol was a product of Parke, Davis and Co.

Results

Most of the data presented in this report are the trichloroacetic acid precipitable cpm present in the high salt soluble fractions of each experiment. For each experiment, the treatments were pooled, and the DNA was extracted (32). It was found for each experiment that the sum of the radioactivities in the separate fractions corresponded to the radioactivity in the DNA of the pooled fraction. Next, all the DNA preparations were analyzed for purity. They all were completely resistant to alkali, and they were all completely degraded to trichloroacetic acid soluble products by DNase (50 μg/ml, 40 min at 30°C). In neutral CsCl, the (H)-DNA always banded coincidently with marker (32)DNA that was known to be pure tobacco DNA (32). From these results, it is concluded that all the radioactivities measured in the high salt soluble fractions of this study are in tobacco DNA.

The effects of attached leaves or stem orientation on DNA synthesis (as measured by thymidine accumulation into DNA in high salt soluble fraction) in stem segments is shown in Table I. In agreement with an earlier report (32), defoliation inhibits DNA synthesis; inversion of defoliated segments results in an even greater inhibition; and exogenous application of IAA to inverted segments more than counteracts the inhibitory effects of defoliation and inversion. The rates of thymidine accumulation into DNA in the foliated and defoliated segments are presented in Figure 1. For both upright treatments (Fig.

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Table 1. Long Term (24 hr) Effect of IAA on Labeled Thymidine Incorporation into DNA in Defoliated, Inverted Tobacco茎 Segments

<table>
<thead>
<tr>
<th></th>
<th>Foliated§ Upright</th>
<th>Defoliated§ Upright</th>
<th>Defoliated§ Inverted 11.9 μM IAA</th>
<th>Defoliated§ Inverted 5.8 μM IAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioactivity incorporated (cpm/g labeled stem tissue)</td>
<td>40,000</td>
<td>29,000</td>
<td>22,000</td>
<td>77,000</td>
</tr>
</tbody>
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§ Leaves removed after isotope incorporation.

1, a and b), there is, in the dark, a similar initial rapid rate of accumulation of thymidine into DNA which starts to decrease after about 1 hr. In the foliated segments, some thymidine continues to accumulate into DNA throughout the remaining incubation period, but in the defoliated segments thymidine accumulation stops completely after about 4 hr. Inversion of the defoliated segments (Fig. 1c) reduces the initial rate of thymidine that accumulates into DNA. The stability of DNA synthesized in foliated explants is shown in Figure 2. It can be seen that, as expected, the addition of a vast excess of unlabeled thymidine stops measurable incorporation of labeled thymidine into DNA. Moreover, since no tritium counts can be chased out of labeled DNA by up to 40 hr of treatment with unlabeled thymidine, the DNA is stable. The results indicate that the rates of thymidine accumulation into DNA in foliated and defoliated segments (Fig. 1) truly reflect the incorporation of thymidine and not net rates of synthesis minus degradation of DNA.

The effect of placing defoliated, inverted segments after uptake of labeled thymidine into IAA solution and then incubating them in the dark is shown in Figure 3. It is clear that there is a striking increase in incorporation of labeled thymidine into DNA 90 min after exposure of segments to IAA and that labeled thymidine continues to be incorporated into DNA at a high rate throughout the incubation period (20 hr). When defoliated, inverted segments are first incubated for 7 hr in the dark so that incorporation of labeled thymidine into DNA stops and then segments are transferred to IAA solution, incorporation resumes within about 60 min (Fig. 4). Thus, the inhibitory effects of defoliation and inversion of segments are not due to degradation of labeled thymidine, and IAA actually initiates incorporation of thymidine into DNA.

The movement of IAA test solution, as measured by 2-(14C)-IAA, in sections of defoliated inverted stem segments, under conditions used in previous experiments, is shown in Figure 5. It can be seen that 60 min (30 min in light plus 30 min in dark) following the initial exposure of an inverted segment to 2-(14C)-IAA, there is a peak of radioactivity in the stem top, and there is measurable radioactivity in every stem section. Progressively longer periods of time in the dark result in enhancement of radioactivity in every section. Wardell and Skoog (32) have found that the minimum concentration of IAA necessary to increase thymidine incorporation into DNA in vegetative tobacco stem segments is 2.8 μM. The cpm of 2-(14C)-IAA in a segment section that correspond to 2.8 μM IAA/section is estimated to be about 60,000. From this value and by assuming that the radioactivity remains in IAA, the number of stem sections in each segment that contain enough IAA (2.8 μM) to increase thymidine incorporation into DNA can be estimated (see dotted line of Fig. 5). The relative areas of these sections are plotted against IAA transport time in Figure 6. When the linear curve obtained is extrapolated to the abscissa intercept, it can be seen that about 35 min are required before there is sufficient IAA in any area of a segment to give an increased thymidine incorporation response. Taking the 35-min IAA transport time into account, the data in Figure 4 suggest that stem tissue already containing at least 2.8 μM IAA should reinitiate thymidine incorporation into DNA within about 25 min.

**FIG. 1.** Time course of thymidine accumulation into DNA in stem segments. Following uptake of labeled thymidine by the 2 segments in each of the 8 to 10 different treatments/experiment, the segments/treatment were placed into 10-ml beakers (1 treatment/beaker) each containing 4 ml of H₂O. Then, each treatment was incubated for a different period of time.

**FIG. 2.** Decay of labeled DNA synthesized in foliated, upright stem segments. After 12 hr of incubation (Fig. 1), the 2 segments in each of 6 treatments of the remaining 12 treatments were placed into 10-ml beakers (1 treatment/beaker) each containing 4 ml of 1.8 M unlabeled thymidine solution (2000× the concentration of labeled thymidine). For uptake of cold thymidine, the treatments were placed in the light for 45 min. Then, the incubation in the dark was continued. H₂O only (●); H₂O followed by vast excess unlabeled thymidine solution (○).
DISCUSSION

IAA treatment of stem segments from vegetative tobacco plants has been known for many years to influence the segments' nucleic acid contents (25, 26). Detailed studies with tobacco pith tissue instead of segments have indicated that physiological concentrations of IAA (≤17 μM) effect increases in DNA and RNA levels before increases in fresh weight of the tissue (24, 25). Low concentrations of IAA appear optimal for increasing DNA content, whereas higher IAA concentrations appear optimal for RNA (24). For DNA, additional studies have shown that concentrations of IAA (11–17 μM) which result in the greatest enlargement of vegetative pith cells cul-

FIG. 3. Effect of IAA on thymidine incorporation into DNA in defoliated, inverted stem segments. The conditions were similar to those in Fig. 1, except that the segments in each treatment were placed into separate beakers containing 4 ml of 11.4 μM IAA solution instead of 4 ml of H₂O.

FIG. 4. IAA-induced initiation of thymidine incorporation into DNA in defoliated, inverted stem segments. The conditions were similar to those in Fig. 1, except that after 7 hr of incubation in 4 ml of H₂O the segments of the remaining 9 treatments were transferred to 4 ml of 11.4 μM IAA solution.

FIG. 5. Transport rate of IAA in defoliated, inverted stem segments.
tured in vitro (25) also increase incorporation of labeled thymidine into DNA (7). Optimum growth of tobacco pith tissue induced by IAA has been shown to occur without cell divisions (15). Taken together the above results suggest that the higher levels of DNA in pith tissue treated with IAA are associated with cell enlargement rather than cell division. Recent support of this conclusion comes from several laboratories (9, 10, 17) in which it has been observed that inhibitors of DNA synthesis block elongation.

Employing an improved method for extracting DNA from tobacco stem tissue, the effect of IAA on DNA synthesis in vegetative tobacco stem segments has been reinvestigated (32). The recent results with segments are in agreement with the earlier work with pith tissue. In addition, a higher than optimal IAA concentration (23 μM) for growth has been found to reduce thymidine incorporation into DNA. This lower rate of thymidine incorporation into DNA may be due to ethylene, since it has been shown in peas that high concentrations of IAA stimulate ethylene production (5), which in turn inhibits DNA synthesis (3, 23).

In all the previous work concerning the effects of physiological concentrations of IAA on DNA levels in vegetative tobacco stem tissue, only long term responses were studied. In this report, the short term changes in thymidine incorporation into DNA are described. The data presented here indicate that IAA very rapidly and strikingly initiates thymidine incorporation into DNA in segments that have completely stopped synthesizing DNA. The short time interval before a measurable response suggests that IAA may increase DNA levels before affecting total RNA (12, 13) and total protein (12, 19, 20). Additional experiments using liquid cultures of single tobacco cells may indicate an even shorter response time for thymidine incorporation into DNA than in the segments.

Thymidine incorporation into DNA in stem segments is clearly decreased by physiological treatments seemingly unrelated to DNA synthesis such as moving segments from the light into the dark, defoliation of segments, and inversion of defoliated segments. On the basis of the capacity of IAA to reverse the inhibitory effects of these treatments, it may be that darkness, defoliation, and inversion reduce auxin levels in stem segments. In vivo experiments with tobacco have indicated that there is transportable auxin in young leaves of plants kept in the light and that there is complete cessation of auxin production in young leaves of plants kept in the dark (4). It is not clear why there is so much less thymidine incorporated into DNA in defoliated, inverted segments as compared to defoliated, upright segments.

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LITERATURE CITED


