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Distribution of Three Auxin Protector Substances in Seeds and Shoots of the Japanese Morning Glory (Pharbitis nil)

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Summary. The existence of substances which inhibit the enzymatic destruction of auxin in shoots of the Japanese morning glory (*Pharbitis nil* Choisy) has been confirmed, as has the fact that these substances are distributed in a gradient diminishing from apex to base in a manner indicating a regulatory role in internode elongation and tissue maturation. In addition to the 2 auxin protector substances reported previously (protectors I and II) which appear to account for most of the inhibition of the enzymatic destruction of auxin in young, elongating stem tissue, a third substance, designated as protector A, has been found to be highly active in seeds, and shoot tips of mature plants: In germinating seeds, no protector I or II activity was observed; in stem tips, no protector II and only slight protector I activity was observed. In contrast, old tissue contained no detectable amounts of protector A, but did contain protectors I and II. Between these extremes along the shoot axis, mixtures of the 3 substances were found. The evidence can be interpreted to mean that protector A is degraded into protectors I and II and perhaps translocated in this form. Gel filtration studies indicate that protector A has a molecular weight exceeding 200,000 gm/mole.

A previous report described the occurrence of substances with relatively high molecular weights, which inhibit the destruction of IAA both by commercial horseradish peroxidase and by enzymes present in Japanese morning glory stems (5). In young morning glory plants the inhibition of enzymatic destruction of IAA varied inversely with the age of the internode tissue, i.e., inhibition decreased as one descended along the stem, and the interference with destruction correlated with the rate of internode elongation in young vines (6). The bulk of the inhibitory activity found in young, elongating internodes could be associated with a substance designated as protector I (molecular weight 5000–10,000 gm/mole). Protector II (M.W. 1500–5000 gm/mole) accounted for a smaller, but significant fraction of the inhibitory activity. A third component with molecular weights exceeding 10,000 gm/mole was also found, but in the elongating internodal tissue studied, this component appeared to account for only a very small part of the inhibition (5). This third component was not studied at the time, nor were any analyses made of plant parts other than stem tissue. It is the purpose of the present communication to report that this third inhibitor of auxin destruction, designated as protector A, exists as a highly potent fraction in seeds and shoot apices. The gel filtration evidence indicates that protector A possesses a molecular weight exceeding 200,000 gm/mole, and the new data can best be interpreted to mean that this protector is degraded into protector I and II during maturation of embryonic tissue.

Materials and Methods

Plants. Tokyo standard type, a wild type strain of the Japanese morning glory (*Pharbitis nil* Choisy) was used throughout this study. The plants were cultured in a greenhouse with supplemental lighting, as described previously (6).

Tissue Extracted. Stem tip: shoot apex including young internodes less than 5 mm in length. Young stem: consisting of young internodes 0.5 to 8 cm in length. Young leaf: leaves with a midrib less than 4 cm in length. Old stem: containing basal internodes exceeding 12 cm in length, and of brown color. Old leaf: leaves with a midrib exceeding 8 cm in length. Seed, 17 hours: seeds were incubated at 22° on moist paper towels in a petri dish for 17 hours. The seed coat and associated mucilaginous substance were then removed and the remaining tissue analyzed. Cotyledon, 2 days: seeds were sown in a moist sand box and after 2 days the swollen seeds were dissected to remove the cotyledons. Cotyledon or hypocotyl, 10 days: seeds were sown in germination flats. After 10 days seedlings were collected and analyzed.

In addition to the above, the individual leaves and internodes of a single plant were also analyzed (table I).

Extract Preparation. Tissue was crushed and ground in a mortar in the presence of sufficient potassium phosphate buffer (0.02 M, pH 6.1) to

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obtain a final concentration range of from 10 to 500 mg fresh weight per ml, depending on the tissue. The debris was squeezed through cheese cloth and the resultant crude extract was centrifuged with a clinical centrifuge at maximum speed for 10 minutes. The supernatant fraction was placed in a deep freeze at −12° until use.

Dextran Gel Filtration. Graded dextran gels, Sephadex G-50, G-75, G-100, and G-200 (Pharmacia Co.) were used to filter the extracts, using techniques described previously (5). Tissue extract (0.5–2.0 ml) was applied to the top of the chromatographic tube (15 × 300 mm). The column filtrate was collected serially in a fraction collector as 3.0 ml samples. Dextran blue 2000 (Pharmacia Co.) and pyronin Y (Allied Chemical), with a molecular weight of about 2,000,000 and 300, respectively, were used as dye markers.

Inhibitor Assay. The general technique for detecting protector substances has been described previously (6). The reaction mixture contained 0.1 mM IAA (Calbiochem), 0.1 mM dichlorophenol (Eastman Organic Chemicals), 0.1 mM manganese chloride (Baker analyzed), 1 μg commercial horseradish peroxidase (Calbiochem, RZ value 0.84), and the extract or Sephadex filtrate, made up to a total volume of 5.0 ml with 0.02 M phosphate buffer, pH 6.1. Samples (0.5 ml) were withdrawn at 15 to 30 minute

Table I. *Auxin Protector Activity in Leaves and Internodes of an Individual Young Morning Glory Vine in Relation to Elongation Over a 3-day Period*

Internode number above cotyledonary node	1	2	3	4	5	6	7	8	9	apex
Internode length at T_0 (cm)	0.8	0.8	2.3	5.5	11.3	9.2	3.3	0.8	...	...
Increase in length at T+3d (cm)	0.0	0.0	0.0	0.0	0.3	2.5	6.9	4.7	1.5	...
Relative inhibitor titer* of internode extract	8–16	16	4–8	2	2	2	8–16	16	32	...
Midrib length (cm) of leaf at suprajacent node (T+3d)	5.7	7.4	7.7	6.6	5.7	3.5	1.9	1.0	...	...
Relative inhibitor titer* of leaf extract	4	8–16	4–8	4	4	32	32–64	64	...	...

* See table II for explanation of this term.

Table II. *Distribution of 3 Auxin Protectors in Extracts of Various Seeds, Seedlings, and Mature Tissues of Morning Glory Vines*

Plant parts extracted	Sephadex used to separate protectors	Total relative inhibitor titer of extract*	Relative strength of 3 protector components** A	I	II
Seed, 17 hr	or G-100 G-200	128	++++	−	−
Cotyledons, 2 days	G-50	128	++++	−	−
Cotyledons, 10 days	G-50	4–8	+	++++	+
Hypocotyl, 10 days	G-50	16	+	++++	+
Stem tip	or G-50 G-200	32	+++	+	−
Young stem	or G-50 G-200	8–16	+	++++	++
Young leaf	G-50	32–64	+	+++	+
Old stem	G-50	1–2	−	++++	+
Old leaf	G-50	1	−	++++	+

* This value was obtained by dividing fresh weight (in mg) of old leaf tissue required to produce a 30 to 60 minute lag in 5.0 ml reaction mixture, by fresh weight of any other tissue required to produce the same lag under similar conditions. That is, the less tissue required to accomplish the same inhibition, the greater the relative titer.

** Since the assay provides information only in terms of length of lag period (which is not a direct indicator of quantity of protector substances present), the figures are given as − (no. lag), and +. . .++++ (increasing lag periods).

intervals and mixed with 1.0 ml Salkowski reagent. The OD of this mixture was measured at 540 mμ with a Coleman Junior Spectrophotometer, Model 6D.

Sephadex filtrate (0.2–1.0 ml) was added to the reaction mixture and the length of lag was used as a parameter of inhibitor strength. The amount of tissue extract, i.e., the mg (fr wt) of tissue per 5.0 ml reaction mixture, needed to obtain a 30- to 60-minute lag was used as an indicator of inhibitor titer.

Results

The results are summarized in tables I and II. They confirm the gradient of auxin protection reported previously (6) except that in the young vine described in table I there is a surprisingly large amount of auxin protection evident in the oldest internodes (No. 1, 2 and 3). From internode No. 4 on up, the gradient is typical of young vines with a sharp increase in protector activity as one ascends to internode No. 7 which is still elongating rapidly. In this plant the leaves were also assayed and the discontinuity in protector activity is even more pronounced at leaf No. 6 above internode No. 6 which is still young and elongating, whereas the next leaf farther down (No. 5) is already approaching maximum size.

The data in table II are not based on the analysis of a single plant, but refer to pooled data on various regions of the morning glory shoot. It shows the characteristic gradient in mature plants with protector activity very high in the stem tip and young leaves, high in young stem, and very low in old parts of the shoot. The highest activity is found in seeds and very young cotyledons, with a dramatic drop as the cotyledons age. The hypocotyls of seedlings contained about as much protector substances as did young stems of mature plants.

An analysis of the type of protector substances found in these various plant parts confirms the previously reported analysis of young stem tissue (5): Sephadex G-50 separation of young stem extract showed that major inhibitor activity was associated with tubes No. 7, 8 (protector I) and the second peak was found around tubes No. 11, 12 (protector II), whereas the auxin-destroying enzymes and the third inhibitor appeared in tubes No. 4, 5 with the high molecular weight dye marker.

In contrast to young stem internodes, stem tip extract showed a very different pattern. Here the greatest inhibitory activity was found to be associated with the high molecular weight dye marker in tubes No. 4, 5, while a second, lower peak appeared in the protector I region in tubes No. 7, 8. Even with Sephadex G-200 separation, this high molecular inhibitor came down with the dye, indicating that the molecular weight of the inhibitor exceeded 200,000 gm/mole. We have designated this inhibitor as protector A.

Extracts of old stem, whose total inhibitor activity always proved to be very weak, contain protector I (major peak) and protector II (the second peak), but no protector A. Comparable results were obtained with leaf extracts. Old leaf extracts had no protector A, while protector I was the major fraction both in old and young leaf extracts.

Extracts of both germinating seeds (17 hr) and very young cotyledons (2 days) had protector A as the only detectable fraction. In contrast, extracts of 10-day old cotyledons and hypocotyls showed a pattern just like that of the extracts obtained from young stems or leaves of mature plants insofar as they contained all 3 protectors, the most active peak corresponding to protector I.

Protector A has a molecular weight greatly exceeding that of the IAA-destroying enzyme fraction found in Japanese morning glory tissue (old stem). The IAA-destroying enzyme fraction migrates through dextran gels in a manner suggesting a molecular weight between 10,000 and 50,000 gm/mole and probably close to the latter figure, i.e., the IAA-destroying enzyme fraction migrates with the high molecular weight dye marker through the Dextran G-50 gel (which retards protector I), but is increasingly retarded by Dextran G-75, G-100 and G-200 gels. In contrast, protector A is not retarded by any of these gels including Dextran G-200.

Discussion

The results amplify our earlier observations that along the shoot axis of mature Japanese morning glory vines there exists a gradient of relatively large molecular weight substances which interfere with the enzymatic destruction of auxin (5, 6). Such a gradient of inhibitors of auxin destruction has also been reported by other workers, e.g., again recently in cotton by Morgan (1, 2). However, only Phipps' (3, 4) observations in tobacco appear to involve inhibitory substances with relatively high molecular weights. In all of the above 3 cases (i.e., Morgan's, Phipps', and our work) the evidence strongly points to the inhibitor systems as having regulatory functions associated with maturation and aging. In the morning glory system the auxin protectors appear to regulate stem elongation.

In the present communication several new observations strengthen this concept, viz., there exists a spatial gradient not only in the stem, but also among the leaves. Furthermore, there exists a temporal gradient in the cotyledons following germination. In addition, gel filtration of the extracts of various plant parts showed that there is also a gradient in respect to the type of protector substance found. Thus protector A, a fraction with a molecular weight exceeding 200,000 gm/mole, was consistently found in the youngest parts of the plants (stem tip or seeds), while it was absent from the oldest parts of the shoot. Between the 2 extremes, in young stem, young leaf, and seedling hypocotyl and cotyledons, protector A occurs in diminished quantities while protectors I and II play a much more prominent role in inhibiting the

enzymatic destruction of auxin. These results suggest that protector A is the form stored in the cotyledons, and protector A is also synthesized at the apices of growing vines. In both instances protector A appears to be degraded into protectors I and II, which may be the form in which these substances are translocated into the stem. Alternatively, one would have to propose a de novo synthesis of protectors I and II, and an inactivation rather than a conversion of protector A. For both protectors I and II, a further diminution of titer is associated with the maturation of stem and leaf tissue.

Many questions remain unresolved. The chemical nature of these protector substances has not been elucidated. Nor is the mode of auxin protection clearly established, although we have evidence which indicates that protector I acts primarily as a poiser, not as a specific enzyme inhibitor. The origin and synthesis of these substances is also not clear although the data presented above are suggestive that protector A is synthesized at the shoot tip. The interaction between auxin, and oxidases inhibited from acting by the protectors in vivo, has also not been worked out. We know that in the morning glory vines, oxidases are found in both young and old tissue (5), but no careful quantitative analyses have yet been made. One cannot be absolutely certain, therefore, that the protector substances automatically override the enzymes, since we have found in other experiments (to be reported elsewhere) that increasing the enzyme concentration will decrease the lag period. Conceptually, the picture is further complicated by the fact that the site of enzyme and/or protector action is not known either at the cellular, or at the tissue level, in the morning glory shoot.

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Literature Cited

1. Morgan, P. W. 1964. Distribution of indoleacetic acid oxidase and inhibitors in light grown cotton. Plant Physiol. 39: 741–46.
2. Morgan, P. W. and W. C. Hall. 1963. Indoleacetic acid oxidizing enzyme and inhibitors from light-grown cotton. Plant Physiol. 38: 365–70.
3. Phipps, J. 1965. La plante adulte de Tabac: Mise en évidence et répartition du système auxin-oxidasique. Compt. Rend. Acad. Sci. Paris 261: 3864–67.
4. Phipps, J. 1966. Le catabolisme auxinique chez le tabac: ses modalités dans la plante saine, et parasitée par le virus de la mosaique. Imprimerie du Commerce, Toulouse, France. 159 pp. illus.
5. Stonier, T. and Y. Yoneda. 1967. Stem internode elongation in the Japanese morning glory (*Pharbitis nil* Choisy) in relation to an inhibitor system of auxin destruction. Physiol. Plantarum 20: 13–19.
6. Yoneda, Y. and T. Stonier. 1966. Elongation of stem internodes in Japanese morning glory (*Pharbitis nil* Choisy) in relation to auxin destruction. Physiol. Plantarum 19: 977–81.