Polar Transport of 1-Naphthaleneacetic Acid Determines the Distribution of Flower Buds on Explants of Tobacco

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ABSTRACT

Upon addition of 1-naphthaleneacetic acid (1-NAA) and benzylaminopurine, flower buds developed on explants from flower stalks of Nicotiana tabacum L. cv Samsun cultured in vitro. At low concentrations of 1-NAA, buds emerged mainly at the basal edge, whereas at high concentrations they developed on the remaining surface. The optimum concentrations for the two groups of buds were 0.45 micromolar and 2.2 micromolar, respectively, and the shapes of the concentration versus response curves were similar. The level of benzylaminopurine in the medium affected neither the shape nor the optimum concentration of these curves. The distribution of the buds over the explants was shown to be caused by polar auxin transport, leading to accumulation at the basal side. First, in the presence of the inhibitors 2,3,5-triiodobenzoic acid and 1-naphthylphthalamic acid, both groups of buds had the same optimum concentration of 1 micromolar 1-NAA. Second, after 6 hours of culture applied 1-NAA had accumulated in the basal part of the explant. In the presence of 1-naphthylphthalamic acid, no transport or accumulation of applied 1-NAA occurred.

Polar transport of auxin is involved in diverse processes such as elongation, tropism, apical dominance, cambial division, and differentiation (5). With regard to the effect of auxin on in vitro growth and differentiation occurring in a polar fashion, most research is focused on the formation of phloem and xylem. Basal growth of callus is known since the work of Niedergang-Kamien and Skoog with tobacco pith cylinders and segments (11). Basal regeneration of buds has been observed in leaf explants of Centaurea erythraea (2), leaf explants of Crassula argentea (12, 13), hypocotyl cuttings from Vigna radiata seedlings (3), bulb-scale explants of Lilium speciosum (19), and excised superficial tissue of Nicotiana tabacum (20).

Such uneven development indicates polar auxin transport. However, with the exception of the superficial tobacco tissue, the explants mentioned above represent complex systems comprising epidermis, cortex, and vascular tissue. Transport of auxin through the vascular bundles may result in asymmetric morphogenesis (13). Thin strips from tobacco pedicles, composed of only epidermis and cortex (18, 21), are therefore more suited for a study of the role of auxin transport on morphogenesis.

In general, polar transport of auxin is assumed to occur through the action of an auxin transport carrier, which is located at the basal end of parenchymous cells (8). Polar auxin transport is blocked by a number of inhibitors. Two well-known inhibitors are TIBA1 and NPA. Both act noncompetitively (4), i.e. they bind to the transport carrier but not at the auxin site. TIBA itself is basipetally transported, whereas NPA is not (17).

Polar transport of auxin results in an uneven distribution of buds on explants if the transport leads to an accumulation of auxin in the basal part. Transport through tissue segments has been measured many times using donor and receiver agar blocks which are put against the tissue (see the review of Goldsmith [5] and the references therein). Unequal accumulation of auxin in tissue is rarely measured. An exception is the report of IAA accumulation in Pinus lambertiana embryos by Greenwood and Goldsmith (6).

To our knowledge, this is the first report in which auxin transport and accumulation are determined in relation to asymmetric bud formation. Tobacco floral stalk explants were used for these experiments. Flower buds were formed either at the basal edge or spread over the whole surface, dependent on the concentration of exogenously supplied 1-NAA (20, 22).

MATERIALS AND METHODS

Plant and Tissue Culture. Plants of Nicotiana tabacum L. cv Samsun were raised in the greenhouse according to Van den Ende et al. (20). Flower buds with the pedicels attached were harvested at anthesis and surface sterilized in 2.5% sodium hypochlorite. Superficial explants, 8 × 1 mm and six to eight cell layers thick, were cut and cultured on MS medium (10), supplemented with 1% agar and 125 mM glucose. Unless stated otherwise, groups of 10 explants were placed horizontally on a nylon mesh (width 180 μm) on 25 mL of medium in a 9-cm plastic Petri dish (15, 20, 21).

In this way, the explants were in contact with the medium containing hormones and inhibitors over the whole length of the cut surface. In the medium, the BAP concentration was 1 μM. The NAA concentration was varied as indicated in each experiment. Also the presence of inhibitors is indicated. At low concentrations of 1-NAA, the buds developed on the epidermis at the basal edge, i.e. the edge which in the original position on the mother plant was most proximal to the main stem. Buds on this edge have been defined as basal buds. At higher concentrations, buds were also formed on the remaining surface of the explant. These have been defined as spread or scattered buds.

Quantification of Results. Bud numbers were scored after 14 or 15 d of culture. The results were presented and evaluated after the logarithmic transformation ln(bud number + 2) (20). The number of explants per treatment varied from 10 to 30. Least significant differences (LSD) at p = 0.05 are presented as a bar.

1 Abbreviations: BAP, benzylaminopurine; 1-NAA, 1-naphthyaleneacetic acid; 2-NAA, 2-naphthyaleneacetic acid; TIBA, 2,3,5-triodobenzoic acid; NPA, 1-naphthylphthalamic acid.
Polar Transport of NAA Determines Flower Bud Distribution

Chemicals and Radiochemicals. NPA was synthesized according to Thomson et al. (17). Agar was obtained from Difco Laboratories, and Murashige-Skoog medium was from Flow Laboratories. All other chemicals were from Merck, except BAP, 1-NAA, and 2-NAA, which were from Sigma. 2-[3H]NAA (specific activity 651 GBq mmol−1) and 1-[3H]NAA were purchased from Amersham. The 1-[3H]NAA was custom synthesized and purified by two TLC systems (chloroform:methanol:acetic acid, 75:20:5 and ethylacetate:chloroform:acetic acid, 55:35:10). The specific activity was approximately 185 GBq mmol−1 at a purity of 98%, as checked by HPLC separation (14).

Radioactive Determinations. In general, explants were cultured on media with labeled and nonlabeled NAA as indicated, either with or without a transport inhibitor. After the incubation the explants were thoroughly rinsed, and samples of five explants were dissolved in 0.5 mL of Lumasolve (Lumac) before radioactivity counting with 4 mL of Lumagel (Lumac) in a Philips PW 4540 liquid scintillation analyzer. The results were corrected for quenching and expressed in moles of NAA (15).

In the transport experiment, groups of 40 explants were incubated on 25-mL culture medium without 1-NAA in a 9-cm Petri dish. They were lying on the medium but placed with their basal or apical end against a block of 5 mL of medium containing the labeled and nonlabeled 1- or 2-NAA. This block had been prepared before in a small 5-cm Petri dish, and was placed in the incubation dish on a plastic disc of the same diameter to prevent leakage of the 1-NAA into the supporting medium. Where NPA was used, it was present both in the block and in the supporting medium.

In the experiments in which transport or accumulation was measured, the distribution in the explant was determined by cutting the explants before radioactivity determination in six slices of 1 mm width with a Mickle gel slicer. These results are presented as a percentage of the total amount of radioactivity taken up by the explant.

RESULTS

Position of the Flower Buds. Explants were cultured on a range of auxin concentrations. At 0.045 and 0.1 μM the tissues stayed alive without any development (Fig. 1). At 0.22 μM, the buds that regenerated were strictly confined to the basal edge. Basal bud formation was maximal at 0.45 μM. At this and higher concentrations buds also developed on the rest of the surface. The number of these spread buds reached a maximum at 2.2 μM NAA. At this concentration, only a few buds developed on the basal edge, and callus was formed. At 4.5 μM, scattered bud formation also declined and callus was formed. At 10 μM only friable callus developed. Clearly, both the final number of buds and their distribution over the surface of the explant depended on the NAA concentration.

In order to examine the role of cytokinin in the distribution of buds, the BAP concentration was varied between 0.22 and 10 μM in the presence of 1 μM NAA. Both basal and scattered bud numbers proportionally increased with increasing BAP concentrations (data not shown). Thus it was clear that the BAP concentration had no effect on polarity in our system.

Effect of NAA Transport Inhibitors on Bud Formation. Minimal, optimal, and maximal auxin concentrations are lower for basal than for spread buds (Fig. 1). Transport of 1-NAA in a basal direction, leading to uneven distribution of 1-NAA, could be responsible for this phenomenon. If so, an interruption of the longitudinal auxin transport would lead to an even distribution of buds at all 1-NAA concentrations. To test this hypothesis, explants were grown in the presence of an auxin transport inhibitor, TIBA or NPA. When TIBA was added to media containing 0.45 μM 1-NAA, an inhibitor concentration of 1 μM led to a significant decrease in the number of basal buds and a pronounced increase in the number of scattered buds (Fig. 2). The total bud number was not affected. This shows that TIBA at this concentration strongly reduced the asymmetry of bud formation without interfering with the extent of bud development.

In the next experiment, explants were grown on a range of NAA concentrations in the presence of 1 μM TIBA (Fig. 3). The results show that both types of buds are formed over a wider range of 1-NAA concentrations than in the absence of TIBA. As a consequence, the concentration versus effect curve for the basal buds is broadened and flattened (compare Figs. 1 and 3). The sharp peak in the curve for the scattered buds is unexpected and cannot be easily explained.

NPA also proved to be effective at a concentration of 1 μM. It completely abolishes the uneven bud distribution observed in its absence (Fig. 4). Basal and scattered buds are now formed over a wider range of 1-NAA concentrations, the scattered buds always being more abundant than the basal ones. NPA, therefore, had a larger effect on bud distribution than TIBA (compare Figs. 3 and 4).

Uptake of NAA. Since auxin transport inhibitors are known to stimulate auxin uptake, presumably by inhibiting efflux from the cells (16), and changes in the uptake can influence the

![Fig. 1. Effect of increasing concentrations of 1-NAA on basal (•) and scattered (○) bud formation, determined after 14 d of culture. For each treatment, 20 to 30 explants were used. Error bars represent the standard deviation (vertical bar) does not apply to total number of buds (>).](https://www.plantphysiol.org/content/98/2/753/F1.large.jpg)

![Fig. 2. Effect of increasing concentrations of TIBA on bud formation at 0.45 μM NAA. Ten explants per treatment. Other information is as in Figure 1.](https://www.plantphysiol.org/content/98/2/753/F2.large.jpg)
physiological response to NAA (15), uptake was measured in the presence and absence of 1 \( \mu M \) NPA at several effective NAA concentrations. The results (Table I) show that the uptake in the presence of NPA was not higher than without NPA, but in fact, as tested by analysis of variance, slightly lower after 48 h (\( P < 0.05 \)).

**Transport of NAA.** If longitudinal transport of 1-NAA was to lead to an unequal distribution of the flower buds over the explant, this should be manifest during the inductive period, i.e. during the first 4 d of culture (15) and maybe already during the 1st d. Therefore, the translocation of 1-NAA was measured in explants that were fed radioactive 1-NAA either at the basal or at the apical end for 24 h. At the end of the labeling period, the explants were sliced and radioactivity was determined in the individual sections (Fig. 5).

In nearly all experimental set-ups, including both 1-NAA and 2-NAA with or without NPA, the distribution of label found after application at one end was nearly linear when the logarithm of the counts was plotted versus section number, as expected for diffusion. The only exception observed was 1-NAA, which, when apically applied, had moved to sections more distal to the application site in significantly higher amounts than upon basal application. This difference disappeared in the presence of NPA, and was also not found for 2-NAA.

**Accumulation of 1-NAA.** To see whether the transport indeed leads to accumulation of 1-NAA when the hormone is applied over the whole length of the explant, radioactivity was measured in different parts of the explant after 6 and 24 h of culture in the presence of 1- or 2-[\( ^3H \)]NAA (Fig. 6). It should be noted that in all treatments the most apical part contained less radioactivity than the rest of the explant. The reason for this is that the original explant is cut starting from the basal side, and that the scalpel leaves the pedicel at the apical end. Therefore, the apical end is thinner and will consist of fewer cell layers. Consequently, 1-NAA uptake will be lower because of the smaller sink size. We accounted for this difference in mass by using the analog 2-NAA, which was taken up by the tissue in exactly the same amount (results not shown), but is not subject to polar transport (7). It is seen in Figure 6 that, indeed, the apical part always contains 5 to 10% less radioactivity than the other parts.

The most compelling evidence, therefore, comes from a comparison of the basal and the middle portions. After 6 h, 1-NAA had accumulated in the basal parts of the explant. In the presence of NPA, this accumulation was almost completely inhibited. 2-NAA was not accumulated, and its distribution was not affected by NPA. The uneven accumulation of 1-NAA in the absence of NPA is not a very transient phenomenon but persists for at least 24 h (Fig. 6).

### DISCUSSION

The flower buds that develop on tobacco explants in the range from 0.1 to 10 \( \mu M \) 1-NAA (15) can be divided into two groups, the buds at the basal edge and those scattered over the rest of the surface. Contrary to the findings of Niedergang-Kamien and Skoog (11) for callus growth on tobacco pith segments, there is no gradient in bud number along the explant at intermediate

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**Table I. Uptake of 1-NAA**

Explants were cultured on a range of 1-NAA concentrations and 37 kBq 1-[\( ^3H \)]NAA in the presence or absence of 1 \( \mu M \) NPA. After 24 or 48 h, radioactivity was determined and uptake was calculated. Each value is the mean of four samples of five explants ± se.

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<thead>
<tr>
<th>Concentration of NAA (( \mu M ))</th>
<th>Uptake of 1-NAA</th>
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<tbody>
<tr>
<td></td>
<td>24-h incubation</td>
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<td></td>
<td>–NPA (+NPA)</td>
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<tr>
<td>0.1</td>
<td>37.55 ± 2.42</td>
</tr>
<tr>
<td>1.0</td>
<td>428.6 ± 14.9</td>
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<td>10</td>
<td>4401 ± 149</td>
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auxin concentrations. The scattered buds were fairly randomly distributed between basal and apical end.

The shapes of the concentration versus response curves for basal and scattered buds are identical, but the curves are shifted relative to each other. This enables us to manipulate the location of the bud formation via the concentration of applied auxin. An almost 100% switch from basal to scattered regeneration can be achieved. This is in contrast to many other systems. For instance, Paterson (12) could enhance polar root development on explants from *Crassula argentea* only from 0 to 30% of the total number of roots developed.

This shift could well be the result of longitudinal transport, occurring at all concentrations of 1-NAA. Further evidence in favor of this comes from the experiments with auxin transport inhibitors. These are widely used either for the indication that polar auxin transport is taking place or as a means of influencing transport. As far as they have been used to influence morphogenesis in tissue culture systems, the results were often not convincing. Paterson (13) found that TIBA stimulated even regeneration of roots but that total regeneration was lower. Van Aartrijk and Blom-Barnhoorn (19) observed that TIBA specifically promoted spread bud formation on bulb-scale explants of *Lilium speciosum* but particularly when auxin was present in high concentrations and spread buds were already more abundant than basal buds. We found here that TIBA can both stimulate spread bud formation and reduce basal bud formation at the same NAA concentration (Fig. 3). Furthermore, the influence of TIBA and, even more pronounced, NPA on the two groups of buds is visible at all NAA concentrations, independent of which group of buds is dominant (compare Figs. 3 and 4 with Fig. 1).

Another line of evidence comes from the experiments in which transport was measured as enhanced translocation of 1-NAA in a basal direction upon application of the hormone at one end of the tissue (Fig. 5). This phenomenon is not found for the analog 2-NAA, which does not enter the transport system (7) and which is virtually inactive with respect to regeneration in our explants. Upon application of 1-NAA over the whole length of the tissue, the transport leads to the observed accumulation of 1-NAA at the basal end (Fig. 6). Accumulation of auxin is mostly a result of transport through vascular tissues (9). Here it is the result of transport through parenchymous tissue, the only tissue present in the explant. To the best of our knowledge, this is the first report on polar accumulation of auxin in such tissues in relation to morphogenetic processes.

It is remarkable that a concentration gradient of NAA occurs in the tissue which is over its whole length in direct contact with the medium, taking up large amounts of hormone. Contrary to the contention of Batten and Goodwin (3) that transport would be of no importance when the auxin is supplied over the whole length of an explant, it is clear from our experiments that transport can be measured in such a situation.

The accumulation in the basal part of the explant, relative to other parts of the explant, would be, from the difference between the two curves in Figure 1, expected to amount to a factor between 2 and 5. The accumulation measured is a factor 2 after 6 h of culture and less after 24 h. Of course, the distribution of the hormone over the outer and inner cell layers is not known. The hormone may well be unevenly distributed between outer and inner cell layers. Since the cells appear to be a very large sink for 1-NAA (15), it is very likely that the concentration in the upper cell layers is lower than in the ones close to the medium. The differentiation starts from cells not far below the epidermis.
(21, 22). Therefore, it can be well envisaged that transport of 1-NAA in the upper cell layers leads to uneven formation of flower buds, while a major part of the hormone, in the lower part of the explant, is not being translocated. This, of course, implies that the actual differences in concentration may be higher than those measured in Figure 6.

The distribution found after 24 h of culture is less uneven than that at 6 h. For formation of the maximal number of flower buds, the presence of 1-NAA in the medium is necessary during the first 4 d of culture (15). Presence of a certain concentration during the 1st d seems to be not enough. However, this does not exclude the possibility that the position of the buds is already fixed at a very early stage. If so, this would imply that the 4-d initiation period can be subdivided into two time intervals: the actual induction phase and a subsequent period during which the primary differentiation centers become committed to further outgrowth. Preliminary results on hormone metabolism indicate that within the first 4 d, the internal concentration of free, i.e. nonconjugated, 1-NAA is highest during the 1st d of culture (1, 14), i.e. during the first phase. The hormone metabolism is currently being studied in more detail.

By exploiting the phenomenon of polar development, one can direct the regeneration process to a limited area of the explanted tissue. This results in a high density of regeneration at the basal side, and an almost perfect control tissue at the apical side. The system was used in this way by Wilms and Sassen (22) for a morphological study of flower bud regeneration. The same approach would be useful in studies of the biochemical processes underlying regeneration.

Acknowledgements—The authors wish to thank Dr. G. van den Ende for his stimulating interest and Dr. G. J. de Klerk for helpful discussions and critical reading of the manuscript.

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