CHAPTER

11

The contribution of auxin and cytokinin to symmetry breaking in plant morphogenesis

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CONTENTS

Abstract

New symmetry relations are generated within homogenous and essentially amorphous tissues when a patterned distribution of specialized cells forms *de novo* during the wound regeneration sequence. The origins of the pattern can be traced to the unique physicochemical properties of auxin and cytokinin and the proteins catalysing their metabolism and transport. Positive feedback effects lead to the accumulation of these otherwise cell-permeant growth substances in some cells and their depletion from others, converting an initially homogeneous distribution into a patterned variation in concentration. Supporting evidence is provided from the results of original unpublished experimental work on the patterns of xylogenesis in storage pith of jerusalem artichoke tuber and cytokinin habituation in stem pith of tobacco.

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INTRODUCTION

A most ingenious paradox
W.S. Gilbert

In development, new features emerge from groups of cells in which those features did not exist before in any recognizable form. They arise like Melchisedech without apparent antecedents and their 'spontaneous generation' is compelling. The most hypnotically fascinating examples are those in which a group of identical cells, mitotic products of the same parent cell, sort themselves into different types. Two examples of this sort of symmetry breaking will be considered later. That such a group of cells should acquire increased complexity as a completely autonomous process, independent of outside influences, seems to contravene some cybernetic version of the first law of thermodynamics. However, appearances are deceptive. Complexity is not actually being created *sui generis* but only interconverted. One form of complexity, the genetic information, is being translated into another, the physical phenotype that is the expression of that genetic information.

So much is dogma. The paradox lies in the fact that the base sequence is only one-dimensional whereas the organism into which it is translated is three-dimensional. At the level of a single cell the disparity is not that hard to reconcile if we focus on the process of automatic folding of nascent polypeptides, spontaneously converting the one-dimensional sequence into three-dimensional objects. From here, by way of the specific contribution of each of many proteins to the cytoskeleton, it is a surprisingly short step to the control of cell shape. However, if we move up to a large scale and consider groups of cells, the control system has to operate over a commensurately larger scale, coordinating the responses of many cells, which places it some considerable way further down the line of command from the molecule of DNA. This in turn means that the black box the geneticist has to invoke for delivering phenotype from genotype is even bigger and blacker than usual, and so has room inside it for a biochemist. To the biochemist, this black box is like a rathole to a Jack Russell terrier, even to the extent that it may be difficult to persuade him or her to extricate themselves long after it has been conclusively demonstrated that the rat is no longer at home.

When individual cells within a group become different from each other (the unit of differentiation is the cell), they can do so only if they influence each other which, for plant cells, invariably seems to involve diffusion of hormonal signals across the intervening cell walls.

Hormone and receptor are male and female, mobile versus passive, cell-pervasive versus cell-autonomous, locking in everlasting duality. Over the years, from Alan Turing (1954) to Louis Wolpert (1971), the perceived status of the signal has waned as more and more examples have pointed up the overriding importances of sensitivity, of the cell-specific nature of the response, of changing patterns of responsivity in the responding cells. It is our contention that the distribution of control between the signal and the responding cell is not constant, but is different for different responses. While in the majority of cases the responding cell may well contribute an overwhelming proportion of the total control, there are circumstances in which the level of signal is decisive. One such occasion is in symmetry breaking, when the progressive

amplification of a tiny, initial inhomogeneity is focused by feedback between cells and culminates in its orderly resolution as a sharp prepattern of morphogen.

THE MODEL

To build up a large difference between cells as a result of amplifying a tiny difference in signal strength requires positive feedback processes such as autocatalysis. The classical example is the reaction-diffusion model of Gierer and Meinhardt. There are four component processes (Fig. 1). Activator A activates its own production (process 1), and stimulates the production of inhibitor I (process 2), which suppresses activator A production (process 3) and diffuses much faster than activator A (process 4). Processes 2 and 3 constitute a negative feedback loop, tending to bring any uniform increase or decrease in the concentration of either A or I back to the original concentration. Processes 1 and 4 will amplify any local increase in the concentration of A because the inhibitor produced in response diffuses away faster and suppresses activator production in the area outside that of local activation. Within the area of local activation, the positive feedback effect of process 1 tends to increase the concentration of A, and a stable, non-uniform distribution of A and I is generated (Meinhardt, 1974).

THE EXAMPLES

Over the years, we have experimented with two plant tissue culture systems in which an initially homogeneous set of cells of the same type sort themselves out into a mixture of two different types under the influence of a plant growth substance. We think that in these examples the mechanism for amplifying differs from the reaction-

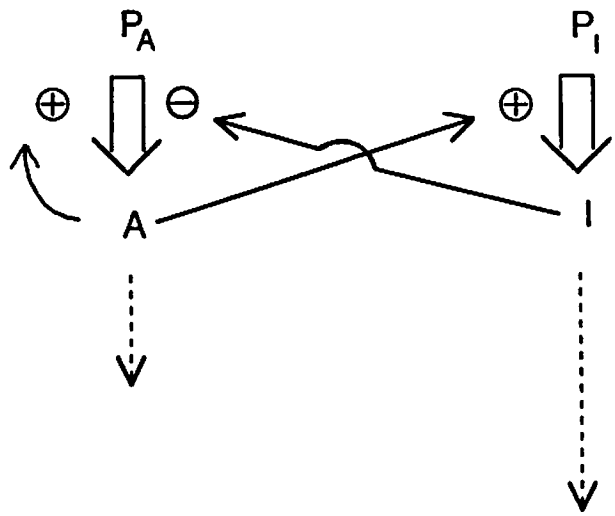

Figure 1 Schematic diagram of the reaction-diffusion model. A is activator, P_A its precursor; I is inhibitor, P_I its precursor. Solid arrows denote stimulation (⊕) or inhibition (⊖). Dashed arrows denote diffusion (after Meinhardt, 1974).

diffusion model in several interesting ways. It seems that control of the intercellular movement of growth substances may play a much more important role than the control of their synthesis, and there is no absolute requirement for the inhibitor, an economy to be expected in view of the constraints of evolution.

Auxin-induced xylogenesis in jerusalem artichoke tuber pith

For the past 15 years we have run an undergraduate practical in which cylindrical tuber pith explants (1 mm high, 4 mm diameter) of the Yeoman strain (Yeoman & Evans, 1967) of *Helianthus tuberosus* L., 'Bunyard's Round', are incubated on nutrient medium containing a xylogenic concentration of indol-3-ylacetic acid (details as in Dalessandro, 1973). The hypertonic medium shrinks the appressed surface of the explant, distorting the explant; and auxin and nutrients diffuse in via its lower perimeter which forms a ring of contact with the medium. Seven days later a network of xylem has differentiated under the surface, within an outer layer of newly formed wound callus (Fig. 2A).

As shown for *Coleus* stem pith (Comer, 1978), this morphogenesis involves two component processes, cell division followed by cell differentiation. Division is initiated in cells situated a few cell diameters below a cut surface, generating a continuous sheet of dividing cells. Auxin is required and, by implication, putative volatile regulators that enable the proximity of the cut surface to be sensed. The pattern of cell divisions is therefore guided by the position of the cut, enabling tissue regeneration to follow the unpredictable topography of a damaged surface. Within the sheet some, but not all, dividing cells differentiate as wound vessel members.

How is it that some of the dividing cells differentiate, but not their neighbours on either side? Xylem differentiates as strands and networks within the continuous sheet of dividing cells. How is this pattern ensured and mass differentiation of all the cells in the sheet avoided?

There is a separate and specific requirement for auxin in xylogenesis, over and above the requirement for auxin in initiating the preceding cell divisions. The clearest evidence is that in some tissues, auxin treatment specifically induces xylogenesis without prior division (Fukuda & Komamine, 1980). We can explain the pattern of xylem if it is prefigured by local accumulation of xylogenic levels of auxin in strands and networks with a complementary pattern of interstrand depletion of auxin, and there are good reasons for predicting that just such a pattern will develop spontaneously as auxin diffuses across a uniform array of otherwise identical cells.

By stimulating H^+-extrusion from cells, auxin promotes its own uptake and transport (evidence reviewed in Goldsmith, 1977), the autocatalytic mechanism for a positive feedback effect. As auxin diffuses across a uniform field of cells, those cells which by random chance contain slightly more auxin than their neighbours at the same position in the gradient will tend to acquire it from them, strengthening their ability to accumulate more. The down-gradient neighbours of such privileged cells may well be disadvantaged relative to this upstream process, but will enjoy an advantage relative to their neighbours at the same position in the gradient, *usw.* The system is analogous to a sand tray demonstration of the origin of water courses and, as for a sand tray, a 'drainage tree' pattern (Fig. 2B) involving progressive capture might be predicted. Similar patterns can be obtained more quickly by spreading oil paint on a piece of glass, covering it with non-absorbent paper and peeling off the paper in

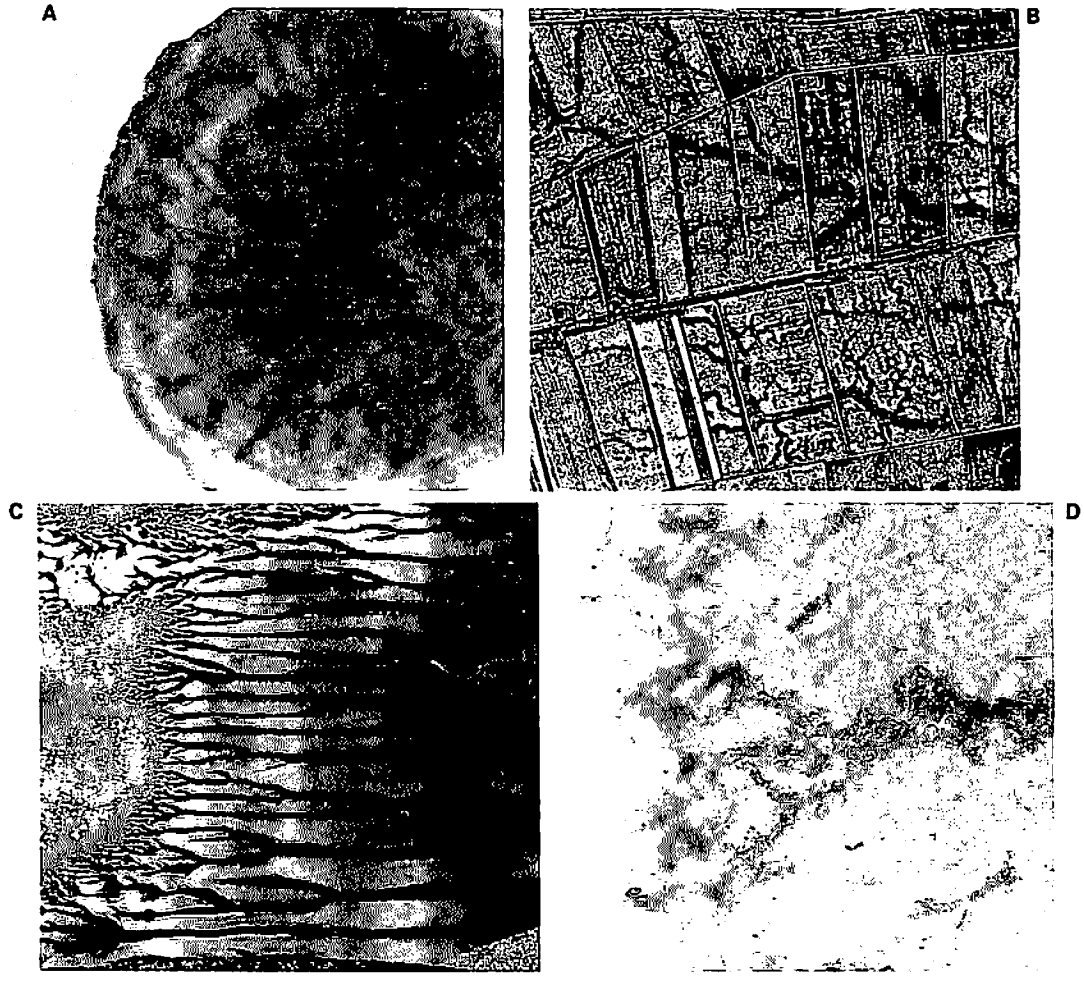

Figure 2 **A** Low-power micrograph of a 4 × 1 mm jerusalem artichoke tuber pith explant after 7 days of incubation on a xylogenic concentration of auxin, cleared and stained with safranin as in Dalessandro (1973). **B** Negative print of an aerial photograph of a fossilized creek system. Silt in a former marshland drainage system shows up light (dark in this negative) against dark fenland soil (light in this negative). **C** Painting by Pery Burge. **D** Micrograph of the cleared and stained result of an experiment in which an auxin gradient and a cytokinin counter-gradient were set up across a thin sheet of jerusalem artichoke tissue. The source of auxin was at the left, the source of cytokinin far to the right, of the region of the sheet depicted here. The frontispiece shows a coloured version of this figure.

one direction (Fig. 2C). In this case, surface tension (the high chemical affinity of oil for oil relative to glass and paper) is the basis of positive feedback.

As a simple test of this idea, we generated an artificial gradient in auxin concentration across a sheet of jerusalem artichoke tissue. Using the techniques and media of Dalessandro (1973), a sheet of tuber pith 50 mm × 20 mm × 1 mm was laid on growth regulator-free nutrient medium. A 35 mm × 5 mm × 1 mm slip of 10 g l^{-1} agar, 5 μM naphth-1-ylacetic acid (a xylogenic concentration), was laid on top of the tissue across one end (Fig. 3A). After 14 days in the dark at 25°C, the tissue was cleared and stained. Wound vessel members had differentiated as scattered groups and individuals in callus that formed on the explant in the area all around the agar slip. No 'drainage tree'

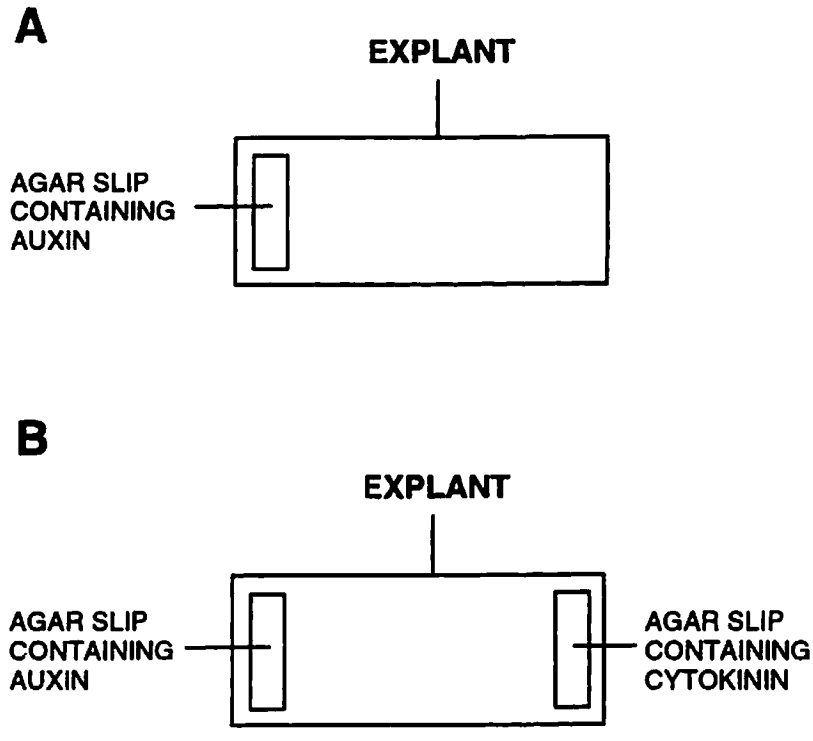

Figure 3 Schematic diagram of the arrangement of agar slips on a sheet of jerusalem artichoke tuber pith tissue in an experiment designed to set up: **A** a diffusion gradient of auxin, **B** a gradient of auxin with a counter-gradient of cytokinin.

patterns were seen. Reasoning that we might be able to enhance the difference between high auxin-containing and low auxin-containing cells by providing a counter-gradient of an auxin-antagonist, we repeated the experiment but added an agar slip of 5 μM kinetin, placed on the upper surface of the explant at the opposite end to the auxin source (Fig. 3B). The result was the differentiation of strands of xylem running from close to the auxin source a short way towards the cytokinin source. Close to the auxin source, the xylem took the form of many fine strands. Further away from it, the strands joined in typical 'drainage tree' patterns as if captured by larger strands (Fig. 2D).

In that this pattern exactly matches the prediction, we have some justification for continuing to test the model proposed here for the formation of patterns of wound vessel members. We need, for example, to establish the existence of the prepattern of channels of high auxin-level, high auxin-transport capacity embedded in the auxin-impoverished mass of tissue. From these results we cannot say that activator alone can generate the pattern because, although the spacing of xylem is well defined for disc-shaped explants on auxin alone, we could only obtain strands within sheets if a cytokinin was applied to the other end of the sheet. However, it does appear that the autocatalytic basis of positive feedback need not involve synthesis, but could be a result solely of the control of auxin movement by auxin. These, and other ideas on the role of auxin in pattern formation, have been discussed by Tsvi Sachs (1981).

Cytokinin-induced habituation in tobacco stem pith

The second example also involves a set of uniform pith cells sorting themselves out into two different types, in this case: cells stably dependent on exogenous cytokinin for division in the presence of auxin (C^-) *versus* cells stably independent of a supply of cytokinin for division (C^+). The cells in the pith of a tobacco stem at the time it is cut are in neither of these states. Instead they are unstably cytokinin-dependent in that after explantation onto nutrient medium they do not divide in response to auxin unless cytokinin is supplied, but a proportion of them spontaneously become C^+. The process is referred to as habituation, the resulting C^+ types as habituated cells. The cells that do not habituate (always the great majority) do not stay the same; they become C^-. The phenomenon has been intensively investigated by Fred Meins Jr whose research group has characterized various features of habituation, reviewed in Meins (1989). We have been using the system in an undergraduate practical class on radioimmunoassay of zeatin-cytokinins for the past 10 years.

Significantly, exogenous cytokinin at very low concentrations will induce a much higher proportion of the pith cells to become C^+, provided the cytokinin is supplied soon after cutting, i.e. before the cells have become C^- or C^+. The concentration of cytokinin required to promote habituation is an incredible 10^{-10} M, only one-thousandth of that required to induce divisions in the same cells. That supplying a minute amount of cytokinin can bring about a transition to a state in which the cell is independent of any exogenous cytokinin suggests an autocatalytic effect of cytokinin on the internal level of cytokinin, i.e. that the freshly cut pith cells are in a state of readiness to amplify dramatically the tiniest traces of this growth substance. In support of this idea, there seems to be a very sharp threshold for cytokinin-promoted habituation between ineffectually low and maximally effective concentrations, as would be expected for an autocatalytic effect (Meins & Lutz, 1980).

What is the biochemical basis for the autcatalytic effect?

Using our radioimmunoassay for zeatin-cytokinins (Turnbull & Hanke, 1985), we found that both C^- and C^+ lines of callus tissue from stem pith of *Nicotiana tabacum* L. cv. 'Havana 425' had the same, very low levels of zeatin-cytokinins: 1 to 3 pmol zeatin riboside equivalents g^{-1} fresh weight. This means that C^+ lines do not have cytokinin degradation blocked or synthesis stimulated, but instead either the cell cycle block that requires cytokinin for alleviation is now non-functional or the cells are cytokinin-hypersensitive and even the very low endogenous levels are now sufficient to sustain cell division.

For experiments in which we measured cytokinin levels during the early stages in habituation we used a 35°C treatment to increase the proportion of pith cells likely to habituate instead of cytokinin treatment, with a view to avoiding the complications due to exogenous cytokinin. The scheme of the experiments is shown in Fig. 4. Using sterile technique, 5 mm × 5 mm × 5 mm cubes of stem pith were cut from internodes 9 to 15 of 3-month-old greenhouse grown plants of 'Havana 425' and explanted onto solidified Linsmaier & Skoog, 11 μM naphth-1-ylacetic acid, following the standard Meins protocol (Meins & Lutz, 1980) as closely as possible. Control tissue was kept at 25°C in the dark for 14 days and did not habituate (25N). Tissue kept for 7 days at 35°C only revealed whether or not each 5 mm cube had produced habituated tissue after a period of 7 days at 25°C to allow growth. Those showing habituated sectors were dissected into clearly habituated (35H) and clearly non-habituated (35N(−H))

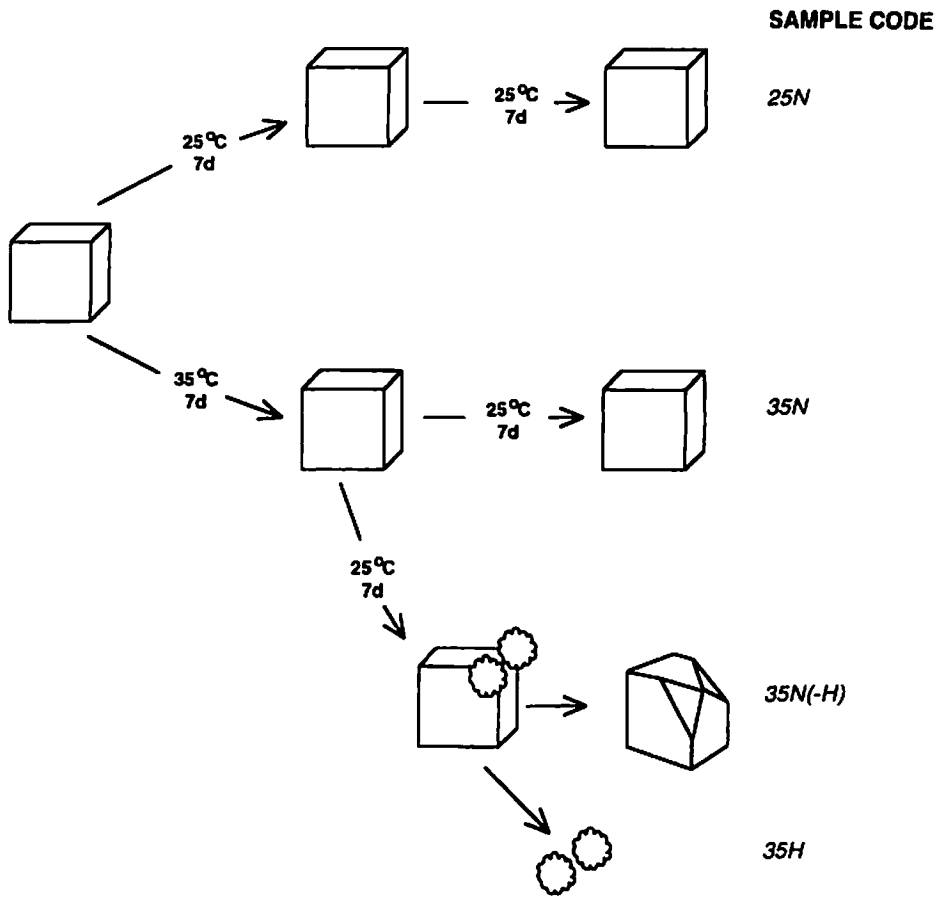

Figure 4 Design of temperature treatments (25°C vs 35°C) and sampling for an experiment to investigate the level of zeatin-cytokinins in cytokinin-independent habituated (H) and cytokinin-dependent non-habituated (N) tissue at 14 days after explantation of pith cubes from tobacco stem on to solidified nutrient medium containing auxin.

samples. Tissue samples were extracted, part purified by SEP-PAK and radioimmunoassayed as described in Turnbull & Hanke (1985). Estimates of the total zeatin-cytokinins in each of these different types of tissue at 14 days after excision are presented in Table 1. Two important points emerge. First, the 35°C treatment does not by itself affect the level of zeatin-cytokinins. If anything zeatin-cytokinin levels fall during culture. Second, C^+ tissue at 14 days contained levels of zeatin-cytokinins up to ten times higher than those in the adjacent C^- tissue of the same pith cube.

It could be argued that the extra cytokinin is a product of the cell division that is restricted to the C^+ tissue in these conditions, but the fact that with time the divisions continue while the excess of cytokinin disappears disproves that idea.

We resolved the zeatin-cytokinins into individual compounds by reversed-phase HPLC (Table 1), and it turns out that almost all of the excess zeatin in the early C^+ tissue is as the ribotide.

Now, cytokinins are very different from most other plant morphogens in their physicochemical properties. The other plant growth substances are hydrophobic but

Table 1 Tissue content of zeatin-cytokinins

Tissue type	Total zeatin-cytokinins before HPLC	Zeatin ribotide	Zeatin riboside	Zeatin free base	Zeatin 9-glucoside
Stem pith at time zero	14	3	0.4	n.d.	0.2
25N	3	2	n.d.	n.d.	0.6
35N	3	5	0.2	4	0.5
35N(−H)	6	8	0.2	n.d.	n.d.
35H	42	25	3	n.d.	4

n.d., none detected.

carry a carboxyl group so that they undergo anion trapping in the cytoplasm of plant cells as follows. Outside the cell they exist predominantly in the neutral hydrophobic protonated form and diffuse across the plasma membrane by passive, non-saturable diffusion – they simply dissolve in and out of the membrane. Once through it, these molecules deprotonate in the mildly alkaline cytosol. Now in the form of a charged anion the growth regulators become lipid-insoluble and must continue to accumulate here so long as the cell continues to pump out the H^+ ions the molecules of growth regulator bring in with them.

Compare this with our picture (Fig. 5) of cytokinin transport at the cellular level as

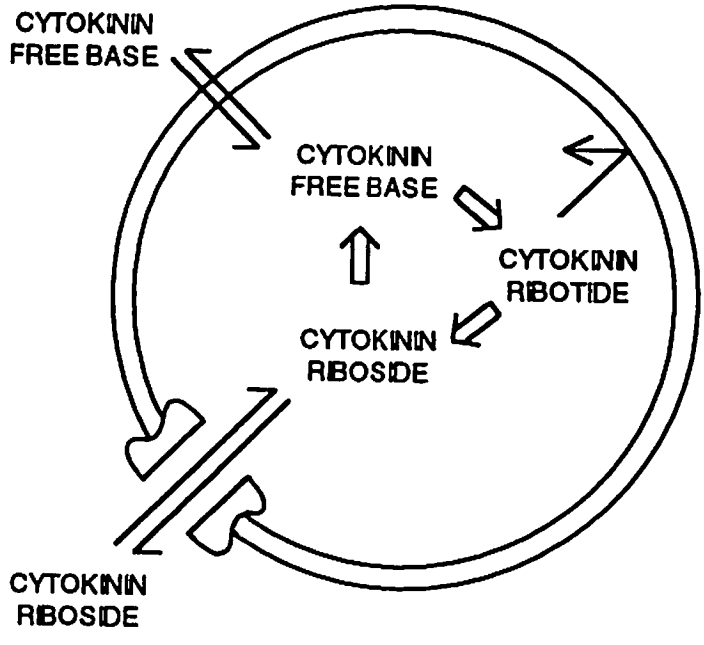

Figure 5 Schematic diagram of the features of cellular transport of the different metabolic forms of cytokinins (after Laloue *et al.*, 1981). The diagram depicts a plasma membrane with one purine nucleoside transporter. Open arrows depict metabolic conversion, solid arrows diffusion.

determined by Laloue *et al.* (1981). In the free base form cytokinins are sufficiently hydrophobic to pass both in and out of the cell by passive, non-saturable diffusion. Once inside the cell they are subject to exactly the same metabolism as the other purines, adenine and guanine, and are efficiently phosphoribosylated to the *ribotide* and in this charged, anionic form they cannot get back out at all. Ribotide is a source of riboside by dephosphorylation. Cytokinin ribosides are free to move in and out of cells, provided the cells concerned have the purine nucleoside transporter in the plasma membrane. The transport of cytokinin ribosides across this membrane is therefore passive but saturable.

It now seems fairly clear to us that the positive feedback mechanism involved in habituation is based on the accumulation of cytokinin, as the ribotide, at the expense of cytokinin picked up from other cells. We do not yet know how. It could be because cytokinins promote phosphoribosylation of purines, or inhibit dephosphorylation of ribotide, or some other connection.

CONCLUSION

Any positive feedback mechanism for symmetry breaking across a group of cells that is built around a molecule that stimulates its own uptake is simpler than one based on a molecule that stimulates its own synthesis. Using synthesis it is necessary to postulate a second component, an inhibitor, to 'talk' to the opposite cell type, rushing out to dampen them down. Using transport the other cells are damped down by being starved of the activator. It seems to us that when both auxins and cytokinins form morphogenic patterns across fields of cells, in both cases the autocatalytic core device at the heart of the positive feedback necessary to amplify signal up to active levels is one based on transport, not synthesis.

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