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DARWIN REVIEW

# The production of auxin by dying cells

**A. Rupert Sheldrake***

Fellow of Schumacher College, Dartington, Devon TQ9 6EA, UK

* Correspondence: rsheldrake@clara.co.uk

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## Abstract

**In this review, I discuss the possibility that dying cells produce much of the auxin in vascular plants. The natural auxin, indole-3-acetic acid (IAA), is derived from tryptophan by a two-step pathway via indole pyruvic acid. The first enzymes in the pathway, tryptophan aminotransferases, have a low affinity for tryptophan and break it down only when tryptophan levels rise far above normal intracellular concentrations. Such increases occur when tryptophan is released from proteins by hydrolytic enzymes as cells autolyse and die. Many sites of auxin production are in and around dying cells: in differentiating tracheary elements; in root cap cells; in nutritive tissues that break down in developing flowers and seeds; in senescent leaves; and in wounds. Living cells also produce auxin, such as those transformed genetically by the crown gall pathogen. IAA may first have served as an exogenous indicator of the presence of nutrient-rich decomposing organic matter, stimulating the production of rhizoids in bryophytes. As cell death was internalized in bryophytes and in vascular plants, IAA may have taken on a new role as an endogenous hormone.**

**Keywords:** Autolysis, auxin, differentiating xylem, dying cells, evolution of auxin, indole-3-acetic acid (IAA), programmed cell death, TAA genes, tryptophan, YUC genes.

## Introduction

Auxin plays many roles in the coordination of growth and development in vascular plants. It was the first plant hormone, or morphogen, to be discovered, and is the most researched. Yet the cellular sites and control of auxin production remain obscure.

The natural auxin in plants is indole-3-acetic acid (IAA). The standard implicit or explicit assumption is that IAA is made in young growing cells in shoot tips, young leaves, and in developing flowers, seeds and roots (Gordon, 1961; Ljung *et al.*, 2002; Normanly, 2010; Brumos *et al.*, 2018). This assumption includes the idea that auxin is made by a regular biosynthetic pathway.

The dying cell hypothesis of auxin production offers an alternative perspective: IAA is a breakdown product of the amino acid tryptophan, the concentration of which increases as cells die and their proteins are hydrolysed (Sheldrake, 1973*a*).

Here, I review the biochemistry of IAA production in the light of recent research and discuss the possibility that dying cells make IAA.

Abbreviations: IAA, indole-3-acetic acid; IPA, indole pyruvic acid; PAT, polar auxin transport.

## The biochemistry of auxin production

The principal biochemical pathway by which IAA is produced within plants is from the amino acid tryptophan. First, the amino group is removed by tryptophan aminotransferases, giving indole pyruvic acid (IPA). Second, IPA is oxidatively decarboxylated by flavin mono-oxgenases to form IAA (Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011; Won *et al.*, 2011; Zhao, 2012). In exceptional circumstances, IAA can also be produced from tryptophan via tryptamine and by several other pathways (Normanly, 2010), but the normal route is via IPA. In experiments with Arabidopsis, genetic constructs that overexpress the genes for these enzymes duly overproduce IAA (Stepanova *et al.*, 2011).

The evidence that IAA is made from tryptophan by this two-step pathway is clear, convincing, and supported by the results of many elegant experiments. As Zhao commented, 'it is quite surprising that plants use a simple two-step pathway as the main mechanism for *de novo* synthesis of the essential hormone auxin, given that auxin biosynthesis was previously considered very complicated' (Zhao, 2012, p. 336).

However, this is not a conventional biosynthetic pathway for several reasons. (i) The efficiency of conversion of tryptophan to IAA is very low, rarely more than 5%, using plant enzyme preparations *in vitro* (Gordon, 1961).

(ii) Tryptophan aminotransferases are non-specific. In species in which their biochemistry has been studied, they work with all three aromatic amino acids— phenylalanine, tyrosine, and tryptophan— and with other amino acids too (Forest and Wightman, 1972; Truelson, 1972; Kutáček, 1985). These enzymes in Arabidopsis are named TRYPTOPHAN AMINOTRANSFERASES OF *ARABIDOPSIS* (TAA), coded for by *TAA* genes, but this name implies a specificity that the enzymes do not have. The principal TAA enzyme, TAA1, also works on phenylalanine, tyrosine, leucine, alanine, methionine, and glycine (Tao *et al.*, 2008).

(iii) The Michaelis constants ($K_m$s) of tryptophan aminotransferases are high; in other words, they are half-saturated only at high concentrations of tryptophan. In maize, the $K_m$ of tryptophan aminotransferase is 300 μM; in peas 100 μM; in tomato, 700 μM (Kutáček, 1985); in mung beans 330 μM (Truelson, 1972); and in Arabidopsis, 290 μM (Tao *et al.* 2008).

(iv) The levels of tryptophan in plant tissues are generally much lower than the $K_m$s of tryptophan aminotransferases. The concentration of free tryptophan in a variety of plant species lies in the range of 1–15 μM (Widholm, 1971). In shoot and root tissues of wild-type Arabidopsis, the level is ~10 μM (Novák *et al.*, 2012; Šimura *et al.*, 2018). These concentrations are determined on a fresh weight basis. Assuming that the water content of the tissues is ~80–90%, then the effective concentration would be somewhat higher—in Arabidopsis ~11–12 μM—but still far below the $K_m$ of tryptophan aminotransferases. Making the unlikely assumption that all this tryptophan is compartmentalized in the cytoplasm, and none in the vacuole, and that the cytoplasm is ~10% of the fluid volume, then the cytoplasmic concentration could be as high as 110–120 μM, still considerably below the $K_m$ of TAA1 at 290 μM.

All viable cells must contain tryptophan because it is a component of many proteins. The biosynthesis of tryptophan, like that of other amino acids, is regulated by the feedback inhibition of enzymes earlier in the enzymic pathway, maintaining a more or less steady-state level. Widholm (1971) found that the key control point in tryptophan biosynthesis was the activity of anthranilate synthetase, the first step in the pathway that leads exclusively to tryptophan. Tryptophan concentrations of 10 μM inhibited the activity of this enzyme by 50%; at 50 μM it was completely inhibited.

The levels of tryptophan within cells must be suitable for the activity of tryptophan-tRNA synthetases, which link the amino acid to tRNA, enabling it to be incorporated into proteins. In yellow lupins, the $K_m$ of this synthetase was found to be 5 μM (Jakubowski and Pawelkiewicz, 1975). Free tryptophan levels of 1–15 μM are in the appropriate range for protein synthesis, but they are far below the levels at which tryptophan is broken down by aminotransferases. If the $K_m$ of tryptophan-tRNA synthetase in Arabidopsis is ~5 μM, then the $K_m$ of TAA1 is ~60 times higher.

(v) IPA, produced by tryptophan aminotransferase, is unstable in aqueous solution and breaks down spontaneously to give several catabolites, one of which is IAA (Tam and Normanly, 1998). Enzymatic oxidative decarboxylation speeds up the production of IAA, and several mono-oxygenases containing flavin prosthetic groups, called YUCs, catalyse the breakdown of IPA in Arabidopsis (Cheng *et al.*, 2006). Other plant species contain similar enzymes. Indeed flavin mono-oxygenases are probably present in all eukaryotic and prokaryotic organisms, and often have broad specificities (Huijbers *et al.*, 2014).

In Arabidopsis at least four different YUC enzymes convert IPA to IAA, namely YUC1, YUC2, YUC4, and YUC6. Plants defective in any one of these genes grow normally, and so do most plants with defects in two of them, although some double mutants are slightly smaller than wild-type plants, as are some triple mutants. However, plants in which all four genes are defective, namely the *yuc1yuc2yuc4yuc6* quadruple mutants, are smaller and have decreased apical dominance (Cheng *et al.*, 2006). However, the very fact that they grow at all indicates that they are still able to make auxin. Direct measurements showed that they contained about half as much IAA as wild-type plants (Stepanova *et al.*, 2011), suggesting either that other enzymes in the YUC family were helping to break down IPA to IAA, and/or that IPA was decomposing spontaneously.

(vi) The non-specific nature of auxin production is shown by the fact that IAA is produced by autolysing yeast and rat liver, as well as by autolysing plant tissues (Sheldrake and Northcote, 1968*a*). In humans, IAA is a non-specific breakdown product of tryptophan. IAA was first isolated from human urine, which typically contains 5–18 mg $d^{-1}$. The amount of auxin in the

urine increases when more tryptophan is ingested (Weissbach *et al.*, 1959).

No doubt in most animal cells, and in living cells in general, the levels of tryptophan are homeostatically maintained at concentrations far below the $K_m$s of tryptophan aminotransferases and other breakdown enzymes. However, tryptophan levels rise as cells die and digest their own proteins. They also rise when animals, fungi, and bacteria digest protein-containing food.

(vii) Crown gall is the exception that proves the rule. There really is a specific biosynthetic pathway for IAA in crown gall cells. Crown gall tumours are caused by the infection of wounds by the bacterium *Agrobacterium tumefaciens*, which genetically modifies the infected cells, causing them to produce both IAA and cytokinin. As a result, they escape from the normal controls over cell division and become cancerous. In tissue culture, they grow in media to which no auxin or cytokinin is added, unlike normal callus culture cells, which require an external source of these hormones.

Two of the genes inserted into the infected plants' genomes code for auxin-producing enzymes that break down tryptophan in a way that normal plant cells do not. The first, tryptophan 2-monooxygenase, catalyses the conversion of tryptophan to indole acetamide; the second, indole acetamide hydrolase, converts indole acetamide to IAA (Thomashow *et al.*, 1986). The *A. tumefaciens* tryptophan 2-monooxygenase has a $K_m$ for tryptophan of 10 μM; the indole acetamide hydrolase has a $K_m$ for indole acetamide of 1 μM (Kutáček and Rovenská, 1991). In contrast, as discussed above, tryptophan aminotransferases have $K_m$s between 100 μM and 700 μM, far above the ambient levels of tryptophan in living cells.

## Tryptophan levels are increased by autolysis

Enzymes of the TAA and YUC types are present in many plant tissues, including meristems, leaf primordia, young leaves, roots, stems, and flowers (Cheng *et al.*, 2006; Poulet and Kriechbaumer, 2017; Shirley *et al.*, 2019). However, it is misleading to think of them as auxin 'biosynthesis' enzymes. Bacteria, fungi, and animals contain similar enzymes and produce IAA when tryptophan levels rise. Tryptophan aminotransferases are broad-spectrum enzymes that break down a range of amino acids; and flavin mono-oxygenases have many metabolic roles (Huijbers *et al.*, 2014). They are not specific enzymes for the biosynthesis of IAA. We humans produce IAA from tryptophan, as do the bacteria in our guts. However, the enzymes responsible are not IAA 'biosynthesis' enzymes; they produce IAA as a result of tryptophan breakdown. We excrete IAA as a waste product. In fact the average concentration of IAA in our own bodies may be higher than in many plant tissues. If a human weighing 70 kg excretes 14 mg of IAA in a day, then the average daily production is 200 μg $kg^{-1}$ FW. For comparison, in Arabidopsis shoots, the highest levels of IAA are in young leaves at ~200 μg $kg^{-1}$ FW, declining to ~5 μg $kg^{-1}$ FW as the leaves mature (Ljung *et al.*, 2001).

TAA- and YUC-type enzymes are necessary for auxin production in normal plants but they are not sufficient. Their presence in plant tissues does not prove that these tissues biosynthesize auxin. They only do so if the levels of tryptophan are elevated. As we have seen, the synthesis of this amino acid is under feedback control from the levels of tryptophan itself. This homeostatic system maintains intracellular concentrations adequate for protein synthesis, but far below the levels at which tryptophan is broken down.

However, *de novo* biosynthesis is not the only source of free tryptophan. Tryptophan levels increase as a result of the hydrolysis of proteins. In plants, there are ~100–1000 times more amino acids in proteins than in their free forms (Hildebrandt *et al.*, 2015). When cells undergo autolysis, the concentrations of tryptophan and other amino acids rise dramatically as proteins are hydrolysed by the cells' own proteases, which are normally located in cell vacuoles (van Doorn, 2011; Olvera-Carrillo *et al.*, 2015).

The release of tryptophan by the hydrolysis of proteins also takes place in living cells that are digesting some of their own proteins, but which are not dying. In differentiating phloem, the nuclei and some of the proteins in the sieve tubes are broken down, but the cells remain alive, though dependent for many of their vital functions on their companion cells (Heo *et al.*, 2017). In the leaves of Arabidopsis plants kept in the dark, partial autolysis leads to dramatic increases in tryptophan levels within 2 d, and even greater increases in longer periods of darkness (Hirota *et al.*, 2018). Autolysis occurs in living as well as in dying cells.

Nevertheless, most of the autolysis in vascular plants occurs as a result of programmed cell death. Protein breakdown in dying cells elevates the levels of tryptophan, which is then broken down by TAA-type aminotransferases giving IPA; YUC-type enzymes then break down IPA to give IAA. These enzymes are widely distributed in plant tissues, but they result in the production of IAA only when tryptophan levels go up. In genetically modified Arabidopsis plants that overexpress TAA- and YUC-type enzymes, more tryptophan released by dying cells can be converted to IAA.

The production of auxin by non-specific enzyme systems in and around autolysing cells may seem too haphazard to ensure a precise control of auxin levels. However, developmental cell death is a controlled, well-regulated process, and levels of free IAA are regulated by enzymes that catabolize or conjugate excess auxin as esters or amide-linked conjugates (Ljung *et al.*, 2002). In maize, tobacco, and Arabidopsis plants genetically modified to overproduce IAA, the levels of IAA conjugates increased up to 50-fold (Tam *et al.*, 2000). In a study with Arabidopsis using a TAA/YUC-overproducing construct, the

concentrations of IAA-aspartate, IAA-glucose, and IAA metabolites increased greatly, but free IAA levels were not significantly different from those in wild-type controls, showing a 'very effective homeostasis mechanism that regulates the levels of free IAA' (Stepanova *et al.*, 2011, p. 3969).

## Dying cells as sites of auxin production

Programmed cell death is an integral part of vascular plant development. All vascular plants, technically known as tracheophytes, contain tubular files of tracheary elements—xylem tracheids—that are able to conduct water because they are dead, empty tubes. After thickening their walls, differentiating xylem cells undergo autolysis and disappear, leaving their tubular cell walls. Fibre cells die as they complete their differentiation. Phellem cells on the outside of the bark die as they differentiate and suberize. As leaves senesce, their dying cells release nutrients that are recycled into other parts of the plant. Cells die during the formation of egg cells and pollen grains. As embryos develop and seeds germinate, a series of nutritive tissues dies, releasing nutrients that are used by the growing plants (Daneva *et al.*, 2016).

In programmed cell death, vacuolar membranes break down and hydrolytic enzymes from the vacuoles digest the contents of the cytoplasm, which is also acidified (van Doorn, 2011; Escamez and Tuominen, 2017). These cell-dissolving hydrolytic enzymes must themselves be relatively resistant to hydrolysis, otherwise these cellular auto-destruct systems would destroy themselves before they catalysed the breakdown of the other cellular constituents.

The dying cell hypothesis of auxin production does not propose that IAA is produced exclusively in dying cells themselves. Some of the tryptophan released by the hydrolysis of proteins may enter adjacent cells and be degraded there. The principal feature of this hypothesis is that auxin is produced as a result of elevated levels of tryptophan released from proteins.

I now discuss auxin production in connection with different kinds of cell death.

### *Differentiating xylem*

Auxin is formed in developing leaves as vascular tissues differentiate. In their classic study, Jacobs and Morrow (1957) found a close correlation between auxin production and xylem differentiation in developing *Coleus* leaves. In Arabidopsis and tobacco, high levels of IAA in young leaves arise as vascular differentiation begins (Ljung *et al.*, 2001). Immunocytochemical techniques have revealed elevated levels of auxin immediately around differentiating vascular strands in leaf veins, where xylem cells are dying ( Avsian-Kretchmer *et al.*, 2002; Aloni *et al.*, 2003), and also around differentiating vascular strands in petals (Aloni *et al.*, 2006) and roots (Aloni *et al.*, 2003; Stepanova *et al.*, 2008; Brunoud *et al.*, 2012).

One of the roles of auxin is the induction of xylem differentiation. If auxin is produced as tracheary elements differentiate, there will be a positive feedback: the more xylem that differentiates, the more auxin it produces, leading to more xylem differentiation (Sheldrake, 1973*a*). This autocatalysis is normally damped down by polar auxin transport (PAT), which carries much of the auxin away. However, if the PAT system is inhibited chemically or genetically, then the auxin released by differentiating xylem cells will accumulate locally, and induce further xylem differentiation, leading to yet more xylem differentiation. In Arabidopsis leaves in which PAT is inhibited, thickened bands of tracheids build up at the leaf margins and in the primary and secondary veins (Mattsson *et al.*, 1999; Verna *et al.*, 2019; Ravichandran *et al.*, 2020). These areas of abnormally enhanced vascular differentiation coincide with high IAA levels, as revealed by immunocytochemistry (Avsian-Kretchmer *et al.*, 2002).

The most impressive accumulations of xylem cells are in in tree trunks. Divisions in the meristematic cambial region bring about secondary thickening by producing new xylem cells on the inside and new phloem cells on the outside. In trunks with active secondary thickening, Söding (1937) found that the cambial region contained the highest levels of auxin. In more detailed studies, the highest auxin levels were in differentiating xylem cells themselves (Sheldrake, 1971*a*; Savidge *et al.*, 1982), or in cells on the xylem side of the cambium (Uggla *et al.*, 1996; Tuominen *et al.*, 1997; Immanen *et al.*, 2016). Smetana *et al.* (2019) used immunocytochemical techniques to study secondary thickening in Arabidopsis roots and detected the highest levels of auxin in differentiating xylem cells, falling off towards the cambium.

The cambial region is the main site of PAT in most secondarily thickening stems and trunks (Sheldrake, 1973*c*; Nix and Wodzicki, 1974), and therefore some of this auxin may have been in transit. However, auxin is also produced within secondarily thickening stems themselves as a result of cambial activity (Söding, 1937; Dörfling, 1963; Sheldrake and Northcote, 1968*b*; Sundberg and Uggla, 1998).

Tobacco internodes grown in tissue culture in the absence of exogenous auxin continued to produce auxin for many months as a result of ongoing cambial activity and vascular differentiation. When PAT was inhibited by tri-iodobenzoic acid, the amount of differentiated xylem greatly increased, with serried ranks of tracheids (Sheldrake and Northcote, 1968*b*), analogous to the effects of inhibited auxin transport on leaf veins in Arabidopsis. These findings suggest that auxin released by the dying xylem cells stimulates cambial activity and leads to more xylem differentiation in a positive feedback loop.

A striking recent study of cambial activity in Arabidopsis roots used genetic contructs to zoom in and actually see where auxin is probably being produced. The plants were genetically modified to express the *DR5rev:GUS* auxin reporter gene, enabling regions in which cells are responding to auxin to be visualized by staining blue. These responses indicated that there were locally

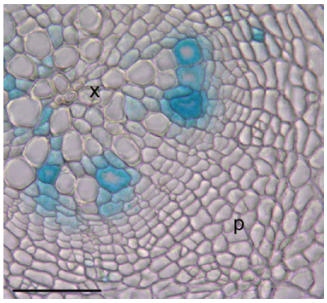


**Fig 1.** A transverse section of a secondarily thickening Arabidopsis root. The blue stain indicates the presence of auxin, as revealed by the auxin-responsive *DR5rev:GUS* marker system. The stain is in and around differentiating xylem cells produced by the vascular cambium, which lies between the xylem (x) on the inside and the phloem (p) on the outside. Scale bar 50 µm. Reprinted from Smetana *et al.* (2019). High levels of auxin signalling define the stem-cell organizer of the vascular cambium. Nature 565, 485–489. Springer Nature, Copyright 2019..

elevated levels of auxin around differentiating xylem elements (Fig. 1). A quantitative study of staining intensity showed that auxin response levels were low in the cambium itself, but increased very significantly as the xylem elements differentiated, rising ~4-fold in the second cell layer from the cambium and ~7-fold in the third (Smetana *et al.*, 2019). In addition, Smetana *et al.* induced xylem differentiation in abnormal positions within the roots, in the phloem parenchyma, by using genetically modified plants that produced scattered ectopic tracheary elements. These differentiating cells, which died as they differentiated, were surrounded by dividing cells, some of which underwent xylem differentiation themselves. The authors described differentiating xylem cells as 'stem-cell organizers' and pointed out that the cambial system is autocatalytic because 'differentiation of the organizer into a xylem vessel leads to formation of a new organizer in the adjacent cambial stem cell, thus ensuring the maintenance of the vascular cambium'. Their observations are in harmony with the idea that differentiating xylem cells produce auxin as their contents break down.

### *Lateral root cap cells*

The root cap forms a sheath around the meristematic cells at the root tip. As the root grows, root cap cells move up the side of the root and die before they enter the root elongation zone (Fendrych *et al.*, 2014). As they do so, they release auxin. In Arabidopsis, the periodicity of lateral root initiation is driven by recurrent bursts of cell death and auxin production by the lateral root cap cells (Xuan *et al.*, 2016). These authors assumed that the principal precursor of IAA was indole butyric acid, but their findings about bursts of auxin production do not depend on this assumption.

### *Endodermis cells*

The endodermis is the innermost layer of the cortex surrounding the vascular tissue in young roots, and also in some stems. It forms a cylinder of cells, the radial walls of which contain a strip of hydrophobic substances, the Casparian strip, restricting the movement of water and ions between the cortex and the vascular system.

In roots, endodermis cells undergo programmed cell death when a lateral root primordium forms immediately inside the endodermis. The death of these cells facilitates the emergence of the new root as it pushes its way through. In genetically modified Arabidopsis plants in which this kind of cell death was inhibited, the emergence of lateral roots was delayed (Escamez *et al.*, 2020). These authors suggested that the breakdown of the endodermal cells might contribute to the growth of lateral root primordia 'by allowing a massive release of cell wall-modifying enzymes and/or of auxin'.

The programmed cell death of endodermal cells also plays a part in the development of the periderm, the protective layer that replaces the epidermis as the outermost protective layer as plants grow. The phellogen, or cork cambium, generates phellem (cork) cells outwards and phelloderm (cork parenchyma) inwards. The release of auxin from dying endodermal cells or wounded epidermal cells seems to play a key role in the initiation of the phellogen (Campilho *et al.*, 2020). The first endodermal cells to die are in front of the phloem poles, and express marker genes associated with developmental cell death (Wunderling *et al.*, 2018).

Phellem cells, which die as they differentiate, also express marker genes for programmed cell death (Wunderling *et al.*, 2018). If they release auxin as they die, they may help to maintain the activity of the adjacent cork cambium, just as the release of auxin by dying tracheary elements may help to maintain the activity of the adjacent vascular cambium.

### *Flowers and developing seeds*

Auxin moves into flower primordia and very young flowers acropetally both through the phloem and by acropetally oriented PAT (Heisler *et al.*, 2005; Aloni *et al.*, 2006). It accumulates at the apical ends of developing sepals, petals, stamens, and gynoecia (Sundberg and Østergaard, 2009). As flowers develop further, auxin is then formed *de novo* both in anthers and in ovules (Robert *et al.*, 2015).

The levels of auxin increase as anthers develop (Aloni *et al.*, 2006; Feng *et al.*, 2006; Cecchetti *et al.*, 2008). Within them, the developing pollen is surrounded by a nutritive tissue, the tapetum. In Arabidopsis, the increase in auxin levels is most pronounced at stages 10 and 11 of flower development, coinciding with the breakdown of tapetum cells (Parish and Li, 2010; Xie *et al.*, 2014).

Within young ovules, the central cellular mass is the nucellus, which gives rise to a megaspore mother cell that undergoes meiosis. In most flowering plants, three of the four haploid daughter cells soon die. These dying cells are at the micropyle, the end of the ovule through which the pollen tube will later enter (Schneitz *et al.*, 1995), and auxin seems to be produced

near the micropyle (Pagnussat *et al.*, 2009; Panoli *et al.*, 2015), perhaps as a result of the death of these cells.

In Arabidopsis, as in most flowering plants, the surviving cell, the megaspore, undergoes three successive waves of nuclear division to produce eight haploid nuclei, all within a single cell membrane. Cell walls then form to produce seven haploid cells that make up the female gametophyte, also known as the embryo sac. Two of these cells are at the micropylar end, and are called synergids; three are at the opposite end, called antipodal cells; two are more centrally located, one of which is the egg cell: the other, called the central cell, has two nuclei that later fuse. The antipodal cells die around the time the flower opens (Larsson *et al.*, 2017).

At fertilization, when the pollen tube enters the ovule through the micropyle, one of the pollen nuclei fertilizes the egg cell; the other fertilizes the central cell, which then divides to form the endosperm, a triploid storage tissue. In Arabidopsis, the fertilization of the central cell somehow sends a signal to the nucellus, triggering its degeneration (Bertoni, 2016). As the nucellus breaks down, auxin levels increase, leading to the growth of the endosperm and the development of the seed coat (Figueiredo *et al.*, 2016; Xu *et al.*, 2016; Larsson *et al.*, 2017). Similarly, in developing apple seeds, the degeneration of the nucellus is correlated with high levels of auxin (Luckwill, 1948). In developing achenes of strawberries, levels of free tryptophan rise to a maximum as the nucellus regresses and large amounts of auxin are produced, stimulating fruit set and the development of the receptacle (Nitsch, 1952).

As seed development continues in Arabidopsis, the cells of the endosperm themselves die; their death is triggered by the mechanical pressure of the growing embryo (Fourquin *et al.*, 2016). In cereal species, the endosperm persists as a storage tissue and occupies most of the seed; the mature embryo makes up only a small part, in barley ~10% (Shirley *et al.*, 2019; the endosperm is broken down and consumed by the developing seedling only after germination.

In an anatomical study of the development of rye grains, Nutman (1939) observed that 'the initiation of each new phase of development occurs by the degeneration of some previously formed tissue and suggested that these regressing tissues supplied hormones as well as nutrients. Hatcher (1943) found that auxin was produced as nutritive tissues regressed in developing rye grains, and suggested that it was derived from the dying cells.

Thus much of the auxin in developing ovules and seeds may be produced by dying cells first in the embryo sac, then in the nucellus, and finally in the endosperm.

In Arabidopsis flowers, auxin is also produced in styles where cells are dying below the stigmatic tips (Aloni *et al.*, 2003). After pollination, pollen tubes grow down the styles, and in some species cause a lysis of the cells between which they pass. In *Nicotiana*, auxin is produced in the apical parts of styles soon after pollination, and then more basally as the pollen tubes penetrate (Muir, 1942; Lund, 1956; Chen and Zhao, 2008).

### *Senescent leaves*

In senescent leaves, levels of free amino acids increase dramatically as proteins break down. In the tips of senescent Arabidopsis leaves, the amount of free tryptophan rose ~20-fold on a fresh weight basis in plants grown in regular light–dark cycles (Watanabe *et al.*, 2013). A similar increase of ~20-fold occurred when Arabidopsis plants were kept in the dark for 15 d, which accelerated the senescence of all the leaves (Araújo *et al.*, 2011). Another study with dark-induced senescence in Arabidopsis found a 320-fold increase in free tryptophan levels (Fahnenstich *et al.*, 2007).

These figures represent a balance between the release of tryptophan from proteins, its degradation by aminotransferases and other enzymes, and its transport out of leaves in the phloem for recycling. In Arabidopsis, >80% of the nitrogen in mature leaves moves out as they senesce (Himelblau and Amasino, 2001). Although some tryptophan may be transported unchanged, much of it is probably broken down by aminotranferases that transfer its amino group into glutamic acid, glutamine, and aspartic acid, which make up the majority of the amino acids in senescent leaves (Diaz *et al.*, 2005) and are also the principal amino acids transported in the phloem sap (Zhang *et al.*, 2010). As tryptophan is broken down by aminotransferases, some of the resulting IPA may be converted into IAA by YUC enzymes, and some may decompose spontaneously.

The leaves of many species show large increases in IAA levels of up to 100-fold as they senesce. Increased levels of auxin in senescent leaves have been found in *Acer pseudoplatanus* (Dörffling, 1963), *Avena sativa* (Sheldrake and Northcote, 1968*c*), *Bryophyllum crenatum* (Raadts, 1962), *Cannabis* (Conrad, 1962), *Cucurbita pepo* (Conrad, 1965), *Hevea basiliansis* (Chua, 1970), *Nicotiana tabacum* (Even-Chen *et al.*, 1978), *Phaseolus vulgaris* (Sheldrake and Northcote, 1968*c*; Wheeler, 1968), and *Prunus cerasus* (Kaska, 1972). In senescing *P. vulgaris* leaves, Atsumi and Hayashi (1979) found that tryptophan levels increased >200-fold and IAA levels >20-fold. There was also a large increase in the levels of IPA, consistent with the breakdown of tryptophan by aminotransferases.

However, Roberts and Osborne (1981) did not observe an increase in IAA levels in the senescent leaves of three species, including *P. vulgaris*. In Arabidopsis, Kim *et al.* (2011) reported that the levels of free IAA fell during dark-induced senescence in detached leaves, but the levels of IAA-conjugating enzymes rose. In contrast, as discussed below, Chen *et al.* (2016) found that free IAA levels increased in detached Arabidopsis leaves, especially when they were kept in the dark.

IAA levels are a result of production, inactivation through IAA-conjugating enzymes, and destruction. In some situations, the conjugation and destruction of IAA in senescent leaves may outweigh its production.

### *Wounded cells*

Cells adjacent to wounded or necrotic areas often react to form a protective layer, usually by cell division below the wound,

with the plane of division parallel to the wound (Bloch, 1952). In this sense, they resemble cambial cells dividing next to dying tracheary elements. In his classic studies on the wound response, Haberlandt (1922) showed that wounded cells released substances that promoted cell division; Hemberg (1943) found that one of these substances was auxin.

Auxin is produced as a consequence of wounding potato tubers (Conrad and Köhn, 1975). Fabbri *et al.* (2000) found that IAA levels doubled within 20 min of wounding. Similarly, sweet potato roots produce auxin as a result of wounding (Tanaka and Uritani, 1979).

No doubt wounded cells release a complex mixture of breakdown products, as do cells undergoing programmed cell death. The same must be true of necrotic cells and cells killed by pathogens. In all these cases, IAA may be one among many breakdown products, but it has a disproportionate effect because plants are so sensitive to it.

In animals too, the breakdown of cell contents in dying cells releases metabolites that act as messengers stimulating wound responses in neighbouring cells (Medina *et al.*, 2020).

## The production of auxin by living cells

Many of the sites of auxin production are sites of cell death. The increased levels of tryptophan in autolysing cells enable TAA- and YUC-type enzymes to produce IAA. Many living cells contain these enzymes and could produce IAA if tryptophan levels rose. How might such increases occur?

The main evidence for auxin production by living cells (apart from tissues transformed by the crown gall pathogen) comes from studies on root tips, young leaves, and detached leaves, as I now discuss.

### *Quiescent centres of root meristems*

Root meristems are the focal points of auxin transport from the whole plant. IAA moves into them both by PAT and by mass flow within the phloem; they accumulate auxin derived from the shoots and made within the roots themselves (Ljung *et al.*, 2005). Auxin is also produced by dying lateral root cap cells, as discussed above. However, on the basis of a series of experiments with complex combinations of mutant genes in Arabidopsis, Brumos *et al.* (2018) persuasively argued that auxin not only accumulates but is also is actually synthesized in the quiescent centres of root meristems. They showed that in mutant plants with defective *TAA*-type genes, the root meristems degenerated, but these mutants could be rescued by genetic constructs in which *TAA*-type genes were expressed in quiescent centre cells. However, the TAA enzymes could only have led to the production of auxin if tryptophan levels were elevated. How could this have happened?

A closer look at the data of Brumos *et al.* (2018) show that what they called the quiescent centre included the cells at the utmost tip of the root, in the columella. The gene expression patterns and auxin levels they studied were manifested both in the quiescent centre cells and a in the columella cells. The stem cells that produce new columella cells are immediately adjacent to the quiescent centre cells, and are closely linked to them (van den Berg *et al.*, 1997). The short-lived columella cells soon undergo programmed cell death (Huysmans *et al.*, 2018). As proteins break down in these dying cells, elevated levels of tryptophan probably lead to the production of IAA within them; and some of this tryptophan may enter the nearby quiescent centre cells, enabling them to make IAA as well.

### *Leaf primordia and hydathodes*

Immunocytochemical methods enable IAA in developing leaves to be localized with high resolution. Elevated concentrations of IAA appear first at the tips of young leaf primordia, later along the margins, and then in the central regions of the lamina (Avsian-Kretchmer *et al.*, 2002; Aloni *et al.*, 2003). Does this mean that auxin is made in the growing cells of leaf primordia, as usually assumed? Or does it move there from somewhere else?

Auxin is probably transported acropetally into leaf primordia and young leaves before vascular differentiation begins (Baker, 2000; Avsian-Kretchmer *et al.*, 2002). In intact plants, auxin travels in the phloem (Allen and Baker, 1980; Baker, 2000; Michniewicz *et al.*, 2007) in which there is an osmotically driven mass flow from sources to sinks. Apical meristems, leaf primordia, and young leaves are sinks; both the phloem and xylem carry substances into them. However, the tips of leaf primordia are many cells away from the apical-most elements of the xylem and phloem. All the cells within these primordia must receive their water, minerals, and organic nutrients through the symplast and apoplast of many intervening cells. Everything moves acropetally from the limits of the vascular strands, presumably including auxin. In addition, IAA is actively transported acropetally in epidermal cells towards the tips of leaf primordia. Unusually, the polar transport system depends on membrane-bound PIN-protein auxin–anion carriers and is similar to the familiar basipteal PAT, except that the PIN proteins are polarized in the opposite direction (Benková *et al.*, 2003).

If young cells in leaf primordia actually produce IAA, then they must contain elevated levels of tryptophan. This cannot come from dying cells, because there are no dying cells in the primordia. Could tryptophan be transported into leaf primordia and young leaves through an active transport system that leads to the accumulation of high levels of tryptophan? No such mechanism is known. An active transport-driven accumulation of free tryptophan may be possible but seems implausible.

As leaves develop further, auxin is concentrated in the veins, and at the hydathodes at leaf tips and on leaf margins (Aloni, 2001; Aloni *et al.*, 2002, 2003; Mattsson *et al.*, 2003).

Aloni (2001) proposed that hydathodes 'are the primary sites of auxin synthesis during leaf development' because so much auxin is found there. However, auxin can accumulate at hydathodes without being made there. IAA travelling in the xylem sap accumulates at hydathodes in coleoptiles and leaf tips of oat seedlings, when they are transpiring or guttating (Sheldrake, 1973*b*), in other words when they are exuding xylem sap under humid conditions as a result of root pressure. The leaves of Arabidopsis also exude guttation fluid through the hydathodes (Aloni *et al.*, 2003). If IAA were present in the xylem sap of Arabidopsis, as it is in oat seedlings and other species (Hall and Medlow, 1974), it would accumulate at hydathodes and leaf margins.

In short, auxin probably moves acropetally into primordia and young leaves from the phloem, and perhaps from the xylem as well, and is then drained out of them by basipetal PAT. There is a kind of auxin fountain. When vascular differentiation begins within young leaves, they start to produce their own auxin, and do so in ever-greater quantities as more leaf veins develop.

Likewise, in flower buds, auxin is at first carried acropetally into the primordia and then basipetally out of them by PAT (Heisler *et al.*, 2005). However, when vascular differentiation takes place within the floral organs, and when the tapetum dies within the developing anthers, developing flowers produce auxin within themselves, as discussed above.

### *Detached leaves*

In studies on the regeneration of roots by detached Arabidopsis leaves, Chen *et al.* (2016) showed that when such leaves were kept in the dark, IAA was produced within the leaves and transported by PAT towards the base of the leaf, where it accumulated in the vascular region near the wound and induced a root to form. This production of auxin began within 2 h of detaching the leaf, and increased thereafter. Much of it seemed to be produced in the mesophyll cells, and was dependent on YUC enzyme systems. These observations are consistent with the fact that keeping detached leaves in the dark accelerates their senescence. Proteins soon begin to break down in cell vacuoles, resulting in increased levels of free tryptophan (Hirota *et al.*, 2018). Chen *et al.* (2016) found that detached leaves kept in the light also produced auxin but more slowly; they were also slower to regenerate roots, as would be expected from the well-known senescence-delaying effects of light.

## The evolution of IAA from a signal of external decomposition to a hormone produced within plants

The earliest land plants were bryophytes, the ancestors of present-day mosses and liverworts (Morris *et al.*, 2018). Their lack of a vascular system restricts their size, and they need humid conditions to grow.

Present-day mosses and liverworts are very sensitive to IAA. They respond to low concentrations of IAA in their environment by producing more and longer rhizoids (Kaul *et al.*, 1962; Maravolo and Voth, 1963), through which they absorb water and nutrients. Rhizoids resemble root hairs in vascular plants, and the sensitivity of bryophytes to IAA depends on genes of 'auxin-response factor' types similar to those found in vascular plants (Cooke *et al.*, 2002; Sakakibara *et al.*, 2003; Thelander *et al.*, 2018). The evolutionary origin of these auxin-response genes can be traced back to the earliest bryophytes (Finet *et al.*, 2012).

Like vascular plants, bryophytes contain *TAA*- and *YUC*-type genes (Thelander *et al.*, 2018), and they also make IAA. However, there is nothing particularly special about having these genes. They are widespread in the plant kingdom, and are present in green and brown algae (De Smet *et al.* 2011; Yue *et al.*, 2014), as well as in bacteria, fungi, and animals. Probably most living cells are capable of making IAA, but do not do so until they die and digest themselves, or are digested by other organisms. IAA is produced as tryptophan levels rise, and these levels rise when proteins break down by autolysis, rotting, and digestion.

I suggest that the stimulation of rhizoid development by IAA evolved in early land plants in connection with the decay of organic matter in their environment. Decomposition releases many nutrients, and there is an obvious selective advantage in producing rhizoids where nutrients are available. IAA could first have evolved as a signal of decomposition.

In samples taken in both tropical and temperate environments, substrata on which bryophytes were growing contained IAA in concentrations from 1 $\mu g\ l^{-1}$ to 50 $\mu g\ l^{-1}$ soil solution (Sheldrake, 1971*b*), sufficient to stimulate rhizoid development in liverworts (Kaul *et al.*, 1962; Maravolo and Voth, 1963). Even in higher plants, exogenous auxin stimulates the growth of root hairs, which are similar to rhizoids (Pitts *et al.*, 2001; Takahashi, 2013), and share a similar gene regulatory network (Jones and Dolan, 2012). This may be an evolutionarily ancient response, perhaps the oldest of all responses to auxin.

This ability to respond to exogenous IAA may have laid the foundations for responding to endogenous IAA when cell death became an integral part of plant development. This process began in liverworts and mosses.

In liverworts, most cells remain alive, but some specialized cells die as the plants develop. In *Marchantia*, a small, flat, dichotymously branching plant, known as a thallus—a plant body without stems, leaves, and roots—there are two principal sites of cell death. The first is in the gemma cups on the upper surface of the thallus, in which small vegetative structures called gemmae develop, which when liberated can grow into new plants, enabling the liverwort to propagate itself vegetatively. These gemma cups also contain mucilage-secreting hair cells, which die after an intense period of secretory activity (Galatis and Apostolakos, 1977).

Secondly, cells die near the junction between the gametophyte and the sporophyte. In liverworts, as in mosses, the gametophyte phase is dominant. These haploid plants produce immobile female gametes and motile, flagellated male gametes. After the eggs have been fertilized by a swimming male gamete, the zygotes develop into diploid spore-bearing sporophytes, which grow out of the gametophytes and are nourished by them. In *Marchantia*, the base of the sporophyte has a foot which contains specialized 'transfer cells' enabling nutrients to be absorbed from the gametophyte, with labyrinthine wall ingrowths that increase the surface area of the cell membrane. On the gametophyte side of the boundary, there are also several layers of transfer cells that degenerate and die as the sporophyte develops (Ligrone *et al.*, 1993).

Immunocytochemical studies with genetically modified *Marchantia* plants containing auxin-response marker genes have revealed two major sites of auxin production: in gemma cups Fig. 2) and around the gametophyte–sporophyte junction (Fig. 3) (Ishizaki *et al.*, 2012); in other words, in regions where cells die.

Like liverworts, mosses are haploid, produce eggs and motile male gametes, and support diploid sporophytes that bear spore capsules on stalks. Unlike liverworts, mosses start life as extensive photosynthetic threads, called protonemata (singular: protonema), that creep along the ground. When they are growing rapidly in bright light, or stimulated by external auxin in dimmer light (Lehnert and Bopp, 1983; Jang and Dolan, 2011), they form elongated, fast-growing non-photosynthetic threads called caulonemata, from which buds develop and turn into the familiar leafy shoots of mosses, with miniature stems and leaves, and with rhizoids emerging from the stems. These leafy shoots are called gametophores.

Moss gametophores resemble vascular plants in several ways. Their stems and leaf midribs contain dead, empty cells, called hydroids, which conduct water, rather like the xylem. They also contain dead cells called sclereids that may play a mechanical role. Sporophyte stalks also contain dead hydroids and sclereids. A genetic analysis has revealed that the genes involved in the production of hydroids and sclereids in mosses are similar to those concerned with the formation of xylem elements and fibres in vascular plants (Xu *et al.*, 2014).

Studies with the model moss *Physcomitrium patens* (formerly known as *Physcomitrella patens*) using auxin-response marker genes indicate that as the leafy gametophores develop, and as hydroids and sclereids differentiate and die within them, they produce auxin (Bierfreund *et al.*, 2003). There are also high levels of auxin in the mucilage-secreting hairs on the stem (Fujita *et al.*, 2008), which die after a burst of intensive secretory activity (Ligrone, 1986), like the mucilage-secreting papillae in *Marchantia* gemma cups. Damaged cells produce auxin too. In an experiment in which gametophore stems were surgically cut, high levels of auxin, as indicated by auxin-response markers, appeared next to the wounded cells (Fujita *et al.*, 2008).

As the sporophyte stalk develops, hydroids and sclereids differentiate and die within it and it has high auxin levels, like the stem of the gametophore. In later developmental stages, the highest activity of auxin-response markers is near the gametophyte–sporophyte junction (Fujita *et al.*, 2008), where sporophyte transfer cells degenerate (Haig, 2013).

The moss *P. patens* contains genes that are homologous with the *SHI/STY* gene families found in Arabidopsis and other flowering plants, which play a role in the regulation of flower development (Gomariz-Fernández *et al.*, 2017). In *P. patens* plants

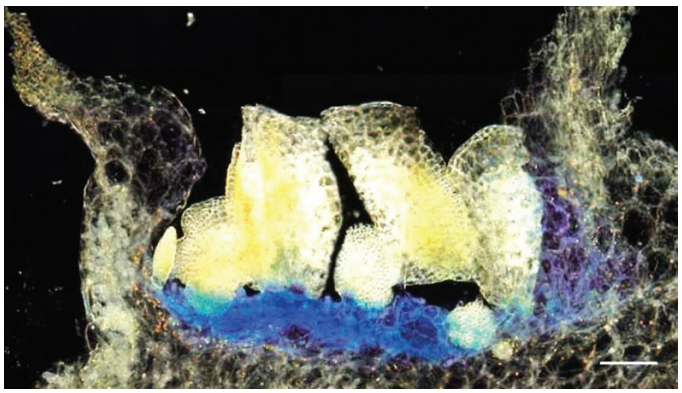

**Fig 2.** A transverse section through a gemma cup of the liverwort *Marchantia*. This transgenic plant contained an auxin-response marker system (*ProGH3:GUS*). The blue stain indicates that auxin was present at the base of the gemma cup, where mucilage papillae die. Scale bar 100 μm. Reprinted from Ishizaki *et al.* (2012). Visualization of auxin-mediated transcriptional activation using a common auxin-responsive reporter system in the liverwort *Marchantia polymorpha*. Journal of Plant Research 125, 643–651. Springer Nature, Copyright 2012.

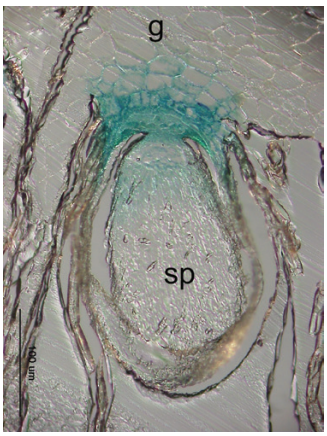


**Fig 3.** A transverse section through a sporophyte with its gametophyte junction (top) of the liverwort *Marchantia* containing an auxin-response marker system (*ProGH3:GUS*). The blue stain indicates that auxin was present around the junction, where transfer cells die. Scale bar 100 μm. Reprinted from Ishizaki *et al.* (2012) by permission from Springer Nature. Visualization of auxin-mediated transcriptional activation using a common auxin-responsive reporter system in the liverwort *Marchantia polymorpha*. Journal of Plant Research 125, 643–651. Copyright 2012.

genetically modified to overexpress similar genes, there was a dramatic increase in the amount of auxin produced. There was also a pronounced necrosis of cells both in the protonemata and in the gametophores. Genetic constructs that caused more cell death led to higher IAA levels than constructs that caused less death (Eklund *et al.*, 2010), suggesting that in these genetically disturbed plants, auxin was produced by dying cells.

Thus, in both liverworts and mosses, auxin seems to be produced as cells die: in liverworts in gemma cups and at the gametophyte–sporophyte junction; in mosses, in the stems of gametophores and sporophytes where hydroid and sclereid cells die as the differentiate, in exhausted mucilage-secreting hairs, and in degenerating transfer cells near the gametophyte–sporophyte junction.

Vascular plants took the evolution of conductive tissues much further with the formation of xylem and phloem elements, and further internalized the production of auxin by dying cells.

## Conclusions

Cells produce IAA by breaking down tryptophan in bacteria, fungi, and animals, not only in plants. Tryptophan levels are elevated by autolysis and cell death. The dying cell hypothesis of auxin production suggests that differentiating tracheary elements are major sources of auxin in stems, leaves, flowers, and roots of vascular plants. Auxin may also be produced by dying cells in root caps, the columella, the endodermis, in regressing nutritive tissues in developing flowers and seeds, and in senescent leaves. The biochemical evidence supports this hypothesis, as does the fact that many of the locations of auxin production are sites of cell death or sublethal autolysis, as in detached leaves. Similar principles seem to apply to liverworts and mosses.

One way of testing this hypothesis would be to look at other kinds of programmed cell death that have not yet been studied from the point of view of auxin production. Do dying cells in abscission and dehiscence zones, in differentiating phellem cells, and in differentiating fibres produce auxin?

Land plants may first have evolved a response to IAA as a chemical messenger from decomposing organic matter, enabling them to produce rhizoids where nutrients were available. IAA may then have evolved into the internal hormone auxin as a result of developmental cell death.

Much of the evidence for this hypothesis is circumstantial, and many questions remain open.

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