PIN-pointing the molecular basis of auxin transport

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Significant advances in the genetic dissection of the auxin transport pathway have recently been made. Particularly relevant is the molecular analysis of mutants impaired in auxin transport and the subsequent cloning of genes encoding candidate proteins for the elusive auxin efflux carrier. These studies are thought to pave the way to the detailed understanding of the molecular basis of several important facets of auxin action.

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Abbreviations

agr	*agravitropic*
ARF	ADP-ribosylation factor
2,4-D	2,4-dichlorophenoxyacetic acid
CW	Cholodny-Went
eir	*ethylene insensitive root*
IAA	indole-3-acetic acid
NPA	naphthylphthalamic acid
PCIB	p-chlorophenoxyisobutyric acid
pis1	*hypersensitive to NPA*
Quercetin	3,3′,4′,5,7-pentahydroxyflavone
rcn1	*roots curl in NPA*
TIBA	triiodobenzoic acid
TIR	transport inhibitor response

Introduction

In 1977, KV Thimann, the grand old man of plant growth hormone research, wrote in his excellent treatise on *Hormone action in the whole life of plants* [1]: "The trouble with auxin is that its actions are so numerous and so apparently unrelated." With more than 70 years of auxin research behind us, what have we learned to tell our students? We have learned much about the chemical identity of auxin and its natural derivatives [2]. We have learned that auxins influence a remarkable variety of developmental and physiological processes [3], but we are still years away from fully understanding the molecular basis of auxin action [4–9].

The application of concepts derived from the study of animal hormones to the design of experiments aiming to unravel the molecular basis of auxin action has not really advanced our understanding of botanical regulation and development. As a result, a controversial debate lasting for many years on the conceptual framework of plant growth substance action was initiated aiming even to abandon the use of the term plant hormone [10–12]. This debate culminated in the suggestion that the activity profile of plant growth substances may be better interpreted as correlative signals — signals that co-ordinate plant growth and development, rather than signals that carry information from source cells to specific target cells or tissues [13]. Applying this conceptual framework to interpret the activities of plant growth substances such as auxin led to the suggestion that auxin might be better viewed as a substance that — similarly to signals acting in the animal nervous system — collates information from various sources and transmits processed information to target tissues [14].

But if auxin does not act like an animal hormone, how can we explain its numerous activities? How can we explain, for example, that auxin can act as a mitogen to promote cell division, whereas at another time its action may be better interpreted as a morphogen [15]? The observation that auxin replaces all the correlative effects of a shoot apex led to the conclusion that auxin is the integrative signal by which the growing shoot tissues influence the development of the rest of the plant [16]. It was also argued that the basic functions of auxin and probably also of the other major growth regulators were to allocate resources under poor growth conditions [13]. The obvious structural similarity of indole-3-acetic acid with the amino acid tryptophan (cytokinin or ethylene share also structural similarities with major metabolites [17]) gave rise to the speculation that plant hormones evolved from more direct, metabolite-mediated signalling systems [17].

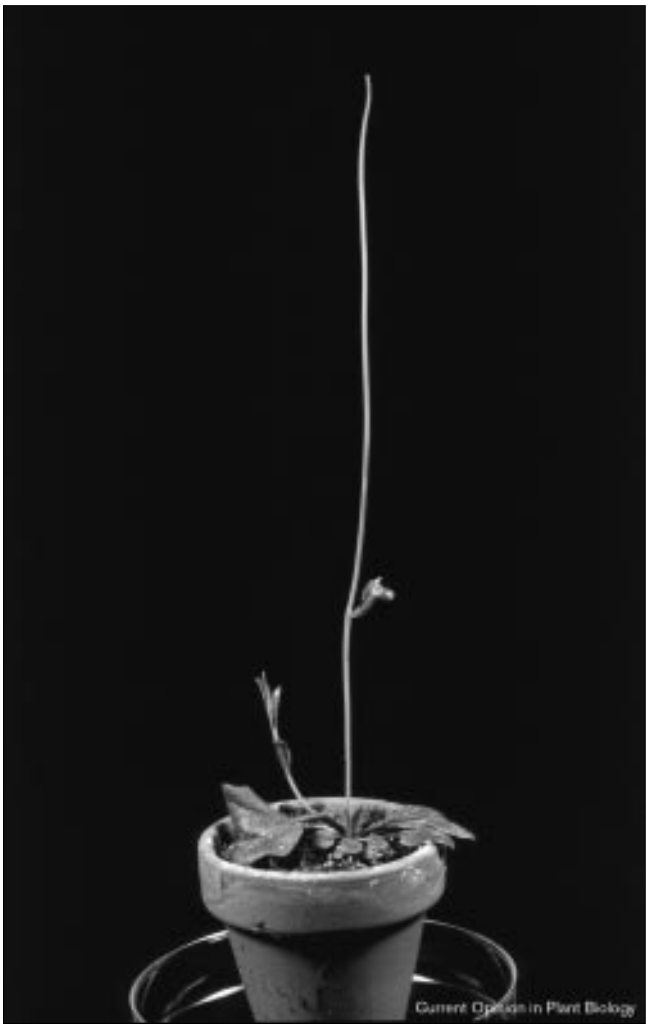
Although this may not be unique as some animal signals (eg serotonin, and other neurotransmitters) also seem to have evolved from metabolites, it is special to plants that auxin acts over long distances and throughout plant life. Hence, understanding the molecular basis of polar auxin transport and how its directionality is established and regulated is likely to give exciting answers as to how auxin acts.

Hence, despite the past and present problems of formulating an entirely convincing conceptual framework for auxin action, we predict that from unraveling the molecular basis of auxin transport the concept of how auxin acts will be illuminated. Indeed, the past decade of molecular and genetic research is starting to bear fruits, already providing significant insights. We will, therefore, concentrate here only on auxin transport, summarise some of the past insights and then dedicate the thrust of this article to a discussion of the auxin transport genes that were considered breakthroughs.

Auxin transport in plant development

Auxin is synthesised in apical growing regions of plants, the shoot apex and the young leaves [18]. From these auxin-producing tissues, the major auxin indole-3-acetic acid (IAA) is unidirectionally transported away towards basally located plant tissues and organs [19]. The transport of IAA through the vascular system of leaves and

Figure 1



The mutation of the *Arabidopsis thaliana AtPIN1* gene changes the phenotypes of *Arabidopsis* plants drastically. The pin-formed, naked inflorescence with no or defective flowers is the main characteristic of these mutants.

stems is polar, occurs basipetally with a velocity of 5–20 cm/h, consumes energy and is specific for biologically active auxins [19]. Pharmacologically active drugs, termed phytotropins, inhibit auxin transport as well as gravitropic and phototropic responses in intact plants [20]. These substances have played an important role in identifying the major elements of the auxin transport system and enabled the selection of mutants. Although the precise mode of action of these compounds is not yet known, they seem to interact with plasma membrane binding sites thereby blocking auxin efflux. In accordance with current models, the basipetal direction of auxin transport suggested a phytotropin and membrane potential responsive uniport system catalysing efflux of auxin anions [21]. Interestingly, the auxin influx was not affected by phytotropins [22].

Polar auxin transport has been linked to the establishment and maintenance of the plant axis [23]. Previous experiments had already implicated that polar auxin transport might be essential for the initiation and maintenance of polarised growth in developing embryos [24–26]. Somatic embryos developing from hypocotyl derived carrot callus cultures were shown to become increasingly sensitive to auxin [26]. Whereas the auxin antagonist PCIB fully arrested embryo development, in the presence of phytotropins, cell division proceeded, resulting in an enormous overall size increase of globular somatic embryos [26].

In later studies, zygotic embryos of Indian mustard (*Brassica juncea* L.) and wheat were successfully isolated and cultured *in vitro* [27–30]. A remarkable range of developmental aberrations was observed in the presence of exogenously applied auxins, auxin antagonists and auxin transport inhibitors. Auxins, such as IAA or 2,4-D, almost completely inhibited the morphogenic progression of zygotic embryos from early radial to late axialised torpedo stages. The morphogenetic changes induced by auxins were mainly enlarged ball- or cucumber-shaped embryos consisting of morphologically different cell layers [30]. In agreement with these observations was the finding that the auxin antagonist PCIB decreased auxin activity resulting in the abolishment of development of one of the two cotyledons at high PCIB concentrations. Auxin transport inhibitors like NPA interfered with cotyledon separation and increased vascularisation in both hypocotyl and cotyledon tissues leading, at higher concentrations, to the formation of a continuous layer of vascular tissue instead of forming individual vascular bundles. Consequently, growing *Arabidopsis* wild-type seedlings on NPA phenocopied the *pin1 (pin-formed)* mutant — a mutant that later turned out to be instrumental to the cloning of the first putative auxin efflux carrier gene [31,32]. Moreover, the observation that quercetin induced a similar range of abnormalities as NPA did in wheat embryo development supported the previously formulated hypothesis that some flavonoids may act as natural regulators of auxin transport [33]. The results from all these reports suggest that auxin is probably synthesised very early in development and that the establishment of auxin transport pathways is a prerequisite to progress from the radial globular embryo stage to later stages with bilateral symmetry. This indicates that auxin is an important initial trigger to induce the developmental steps leading to differentiation of the embryonic axis. The observation, however, that many of the abnormal embryos with multiple meristems and axes induced by inhibition of auxin transport were able to regenerate almost normal plants raises questions as to the importance of changes of symmetry planes in early development. This beautifully illustrates the plastic and probabilistic nature of much of plant construction.

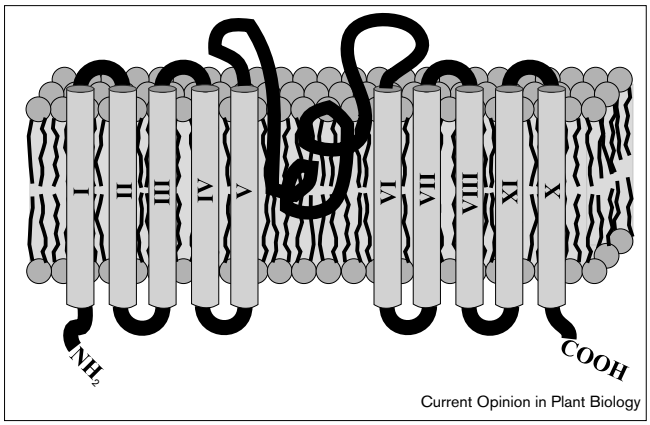
The manipulation of plant development with auxin transport inhibitors also highlighted another important role of auxin — the regulation of vascular development [34].

Early grafting experiments [35] demonstrated that buds induced the differentiation of new vascular tissues and the replacement of buds by auxin indeed clarified that auxin was the sole inductive signal [36]. These classic experiments opened the aera to put forward auxin as a correlative and morphogenetic signal, which probably indirectly affects genetic control of vascular patterning and influences its maturation through a partially self-organizing mechanism [24,37–39]. Sachs reported that the inductive effect of auxin on vascular development extends over large distances throughout the plant [14]. This induction of vascular tissue by developmentally controlled IAA biosynthesis and distribution is peculiar and important for several reasons. Firstly, it specifies the sites where vascular differentiation will occur. Secondly, it determines the direction of vascular tissue formation. Thirdly, it confines the vascular tissue to narrow strands and, finally, it ensures continuity of vascular development and plant growth. The observation that new vessel formation occurs in narrow lines and not in a field is particularly noteworthy when auxin action is conceptually compared to nervous signalling.

A nice illustration of early revascularization after wounding was the analysis of transgenic tobacco plants harbouring the *Athb-8::GUS* gene [40]. This auxin-regulated *Arabidopsis Athb-8* homeobox gene encodes an important regulator of vascular development. It has been suggested that short range signalling mechanisms probably prevent the formation of more vessels within a certain distance from the wounding site [18], but this mechanism has remained elusive to date.

The recent thorough assessment of the role of auxin on the vascular system further strengthened the view that auxin restricts vascular differentiation, promotes its continuity and specifies the leaf venation pattern [41]. The hypothesis that auxin may act more like a morphogen than a cell division promoting factor was directly tested by addressing the role of auxin as a positional signal in xylem development of pine trees [42,43]. The development of accurate mass-spectroscopic techniques for measuring IAA in plants made it possible to measure internal IAA concentrations after application of IAA and/or transport inhibitors in relationship to growth rate and endogenous IAA concentrations. In scots pine, IAA was found to be distributed as a steep concentration gradient across the cambial meristem. The apparent lack of correlation between regions with high IAA concentrations and other regions with high cell division rate provoked the interesting conclusion that IAA indeed provides positional information, rather then acts to stimulate the rate of cell division within the cambial zone [42]. We await with excitement the outcome of future experiments utilising emerging techniques such as genome wide chip-based transcriptome analysis combined with single cell analysis. These experiments will test, at a largely increased resolution, which genes will be activated in response to auxins and which of these genes will ultimately lead to identification of

Figure 2



The AtPIN1 protein is a plasma membrane protein with transmembrane segments and a large hydrophilic loop between transmembrane segments five and six.

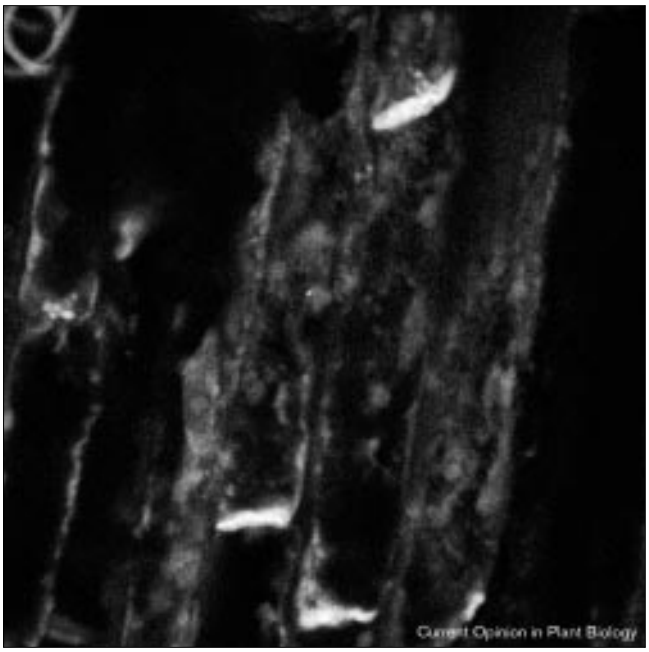
signalling pathways and signal molecules produced in response to IAA. Several other recent reports give further evidence to the view that auxin may play a more general role as a correlative signal — probably conveying information about the presence, developmental rates and location of developing shoot tissues [41–46]. The availability of several relatively easily accessible systems for genetic analysis will soon allow the precise dissection of the molecular pathways leading to formation of the plant axis, and the exploration of the mechanisms that orient cell growth, that control vascular differentiation and, of course, polar auxin transport itself.

Auxin and tropisms

Tropisms are defined as 'directional responses of a plant organ to a directed stimulus in the environment' [47]. Tropic movements allow the plant to adjust itself or orient its organs in response to its surrounding environment for optimal uptake of energy, nutrients or water. In comparison to turgor driven movements, growth movements are typically slow and result from differential growth within or between organs. Such growth movements are important in both phototropic and gravitropic responses in which, after the perception of the initial physical stimulus (e.g. blue light or gravity), a signal will be mediated into the final growth response. Auxin has been implied as the major signal mediating tropic stimuli. Even as early as the 1920s, the Cholodny-Went (CW) hypothesis was formulated to explain the gravitropic response of plant roots and shoots. This hypothesis proposes that the differential growth rates during the gravitropic response are based on auxin redistribution and altered auxin transport within plant tissues [47,48].

Current models indicate that the gravity signal, for example, is perceived at the root cap and transduced via the meristem to the zone of elongation where, at the lower flank of the tissue (oriented towards the gravity stimulus),

Figure 3



The AtPIN1 protein was localized at the basal end of xylem parenchyma cells. The bright signals are visualized by confocal laser-scanning microscopy of longitudinal tissue sections incubated with anti-AtPIN1 antibodies and secondary fluorescently labelled antibodies.

an increase in the auxin concentration can be found, leading to an overall increase of growth rate of the upper surface and a decrease in the lower surface [48]. An interesting illustration of this process has been the recent cloning of a maize coleoptile K^+ channel-like protein [49]. The expression of this gene followed auxin stimulated growth rate kinetics, upon gravistimulation it followed gravitropic induced auxin redistribution, and thus underpins the importance of K^+ accumulation in turgor regulation during cell elongation. But the CW hypothesis may be far too simple to account for all the events occurring during gravistimulation — for example, it completely fails to explain the temporal and spatial variations in gravistimulated tissues.

A wonderful system for the study of growth rates and auxin effects has been the graviresponding gynophore of the peanut (*Arachis hypogaea* L.) — a plant that not only moves its leaves up and down over a five hour cycle, but also buries its fertilised ovule in the underground before fruit and seed development can occur [47,50]. The gynophore is a unique organ that responds to gravity after fertilisation by growing vertically downwards with a growth rate of approximately 0.31 mm/h throughout the distal 10 mm of the tip [51]. Most of the growth takes place at the 2–5 mm region closest to the tip. Close examination of growth rates along gynophore established that growth rates were not uniform, but occurred in wave-like motions along the upper and the lower side of the organ. IAA was clearly the major factor being basipetally transported in a strictly polar manner; however, substitution experiments with externally applied IAA revealed that IAA did not fully restore growth rates, thus indicating a possible role for other growth regulators (e.g. gibberellins) in modulating and fine-tuning growth rates. Moreover, the observation of wave-like motions may indicate the action of several specific carrier proteins for auxin whose activities may be triggered by a complex and as yet uncharacterised signalling network. A series of other recently published papers further illustrate the importance of auxin transport in light-grown *Arabidopsis* hypocotyls, and the role of temporally early-acting modulators of an auxin-responsive pathway that leads to differential growth [52–54].

Dissecting the auxin transport pathway

Mutants have been essential in establishing what is known so far about auxin action [55–57]. Meanwhile, a large collection of mutants exists that affect auxin transport [57–60]. Although some of the mutants have been selected on the basis of abnormal responses to auxin transport inhibitors (e.g. *pis1*, *rcn1*, *tir*), most mutants with altered auxin transport were selected on the basis of resistance to auxins or ethylene. Other interesting mutants affecting auxin responses were identified in screens for various alterations in plant development [44,46,61–64]. Because of lack of space and extensive discussion of some of these mutants in other reviews, we will mention in the next paragraphs only those mutants that are of direct relevance for the recent cloning of essential components of the auxin transport system.

The auxin influx carrier

The notion that the lipophilic protonated form of IAA (probably the predominating form in the acidic apoplast) can easily cross the membrane of vesicles and cells, whereas the deprotonated IAA anion needs a specific carrier for efflux has been around for a long time [65,66]. However, it is difficult to conceive that IAA simply enters the cells by diffusion, with no further control on its uptake. Saturable auxin influx suggesting an auxin influx carrier was indeed found; however, despite the physiological demonstration of an electrogenic proton symport for IAA, its detailed analysis has always lagged behind that of the auxin efflux carrier as specific inhibitors were only available for the latter [67,68]. A mutation in the *AUX1* gene not only conferred auxin and ethylene resistance to *Arabidopsis* seedlings, but also disrupted root gravitropism [69,70]. The corresponding gene encoded a 485 amino acid long protein that shared significant similarity with plant amino acid permeases, prompting suggestions that this protein might be involved in the uptake of the tryptophan-like IAA [70–72]. The recent demonstration that the membrane permeable 1–NAA, but not IAA or 2,4–D, complemented the agravitropic *aux1* root phenotype thereby bypassing the auxin influx carrier defect elegantly showed that AUX1 most probably acts in root apices as facilitator of IAA uptake [71].

The auxin efflux carrier

The basipetal directionality of auxin transport from shoot apices and young leaves to roots is thought to result from the polar distribution of specialised carrier molecules in the plasma membranes. According to the chemiosmotic hypothesis, auxin is assumed to leave transport competent cells only through the activity of specific carrier proteins that are localised at the basal end of these cells [19]. From a large number of biochemical and physiological experiments at least three polypeptides are proposed to be essential elements of the auxin efflux machinery: first, a plasma membrane localised carrier protein; second, an NPA–binding protein; and third, a labile, probably cytosolic component [19,73]. For many years these proteins have been elusive, but from genetic approaches characterising mutants impaired in auxin transport, several genes have recently been cloned that strongly support the concept of basally localised auxin efflux carriers.

Loss of the *PIN1* function resulted in mutant plants with apices which looked like a knitting needle. The mutant was therefore called *pin-formed* or simply *pin1*. The dramatic morphological aberrations observed in this mutant were easily phenocopied when wild-type *Arabidopsis* plants were grown on media containing the auxin transport inhibitor NPA [31,32,61]. When the mutant was analysed, it was found that inflorescences of the *pin1* mutant showed a drastic reduction in basipetal auxin transport — suggesting a mutation in a gene encoding either an important component of the auxin efflux machinery or of the cellular network regulating its activity. The *AtPIN1* gene was cloned by transposon tagging and found to encode a 622 amino acid protein with up to 12 putative transmembrane segments that shared similarities with prokaryotic and eukaryotic transporters [32,74]. Using antibodies raised against a portion of AtPIN1 the protein was found to be localised to the basal end of elongated parenchymatous xylem and cambial cells of *Arabidopsis* inflorescence axes [32]. The polar localisation was confirmed by immunogold labelling and electron microscopy. This localisation nicely confirmed earlier hypotheses and models on polar auxin transport.

An important role of AtPIN1 during early embryo development has been established (Steinman *et al.*, unpublished data). Dynamic changes of AtPIN1 expression were observed gradually narrowing down to provascular cells, which accumulate AtPIN1 at their basal boundary facing the root pole. GNOM, a guanine-nucleotide exchange factor for small GTPases of the ARF family, was found to be important for coordinated polar localisation of AtPIN1 in embryogenesis. The gradual establishment of a polar AtPIN1 localisation from a non-polar orientation along the apical–basal axis during embryo development further suggested a feedback loop involving auxin and indicated that canalization as suggested by Sachs plays an important role during formation of the embryonal axis [36].

AtPIN1 belongs to a family of related sequences in *Arabidopsis* that mostly differ in a hydrophilic region interconnecting two blocks of transmembrane segments. The reverse genetic characterisation of another member of this family, *AtPIN2*, led to the identification of an agravitropic mutant displaying severe defects in root growth and gravitropism [75]. This mutant was shown to be allelic to other known mutants affecting root gravitropism (*wav6-52*, *agr1*) and ethylene sensitivity (*eir1*), leading to parallel studies cloning these genes [76–78]. The agravitropic phenotype was nicely visualised by the absence of the AtPIN2 protein in cortical and epidermal cells [75]. As predicted by the CW hypothesis, auxin, supplied from the shoot, is transported acropetally through the stele in the meristematic region of the root tip where it should be redistributed into the cells of the root cortex and the epidermis and basipetally transported towards the elongation and differentiation zone. The observed preferential localisation of AtPIN2 to the anticlinal basal side of epidermal cell files oriented towards the elongation zone strongly supported the concepts formulated in the CW hypothesis [75].

Moreover, the absence of the *AtPIN2* function not only caused an agravitropic phenotype consistent with a loss of an auxin efflux carrier function in root cortex and epidermis cells, but also resulted in an auxin sensitive phenoytype. Exogenously applied auxin probably accumulated to toxic levels thereby inhibiting further root growth. The other members of the *AtPIN* gene family are currently being characterised in detail (K Palme *et al.*, unpublished data). The available data fit nicely with existing models and also indicate that the auxin efflux machinery is regulated by cellular signalling network involving ethylene, protein turnover and protein phosphorylation and dephosphorylation [59,76,79]. The existing mutants and genes now provide an ideal framework to dissect this network genetically and to study the physiological consequences of auxin transport on tissue organisation and cell differentiation. The mutants described here illustrate the power of the combined genetic and molecular approach, which ultimately will allow us to address the question whether auxin efflux carriers indeed represent the elusive auxin receptor(s) [80].

Conclusions – perspective

Plants — as sedentary — organisms had to develop efficient means of sensing environmental cues, but their ability to respond to these cues was limited by the physical constraints of the plant body. It is the control of organ reorientation, exemplified by tropisms and nastic movements that position these organs for optimal competition in the environment. And it is the auxin which when delivered at the right time to the tissues that control the differential growth responses — elicits these responses. By combining genetic, molecular and physiological technologies we are arriving at the identification of genes and networks acting to control differential growth processes. As Tsvi Sachs, a former PhD student of Thimann, wrote to us recently: "The auxin story is a great illustration of the role of

preconceived ideas in science – and how we refuse to accept results that are not what we looked for. This makes the recent molecular achievements all the more exciting."

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