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**IN THIS ISSUE**

# Plant PtdIns 3-Kinase Goes Nuclear

Phosphoinositides are dynamic and vital members of the cell's repertoire of signaling molecules. They are involved in regulating diverse processes, such as cytoskeletal organization, membrane trafficking, and ion transport (for review see Drøbak, 1992; Munnik et al., 1998; Stevenson et al., 2000). The functional diversity of the polyphosphorylated inositol lipids is in part predicated by the stereospecificity of the phosphate groups on the inositol ring and by the subcellular localization of the phospholipids. In eukaryotic cells, the multiple phosphorylated isomers and the specific lipid kinases involved in their synthesis, (phosphatidylinositol [PtdIns] 3-kinases, PtdIns 4-kinases and PtdInsP kinases), are located in various intracellular compartments, including the plasma membrane, endomembranes, the cytoskeleton, or the nucleus.

The PtdIns 3-kinase family comprises three classes of enzymes that phosphorylate inositol phospholipids specifically at the D-3 position of the inositol ring (Wyman and Pirola, 1998). Both the class I and class II PtdIns 3-kinases are an integral part of receptor-mediated signaling pathways prevalent in animal cells. In contrast, plants and yeast contain only class III PtdIns 3-kinases. The class III PtdIns 3-kinases are typified by the yeast Vps34 protein (Stack and Emr, 1994). In temperature-sensitive mutants of Vps34p, proteins normally targeted to the vacuole are misrouted (Stack et al., 1995), implying a role for PtdIns 3P in vesicle trafficking. Class III-type PtdIns 3-kinase genes have been isolated and characterized from soybean (Hong and Verma, 1994) and from Arabidopsis (Welters et al., 1994). Previous reports in plants have supported a potential function for the PtdIns 3-kinases in vesicle trafficking and vacuolar sorting similar to yeast (Matsuoka et al., 1995).

In this issue of THE PLANT CELL, **Bunney et al. (pages 1679–1688)** provide a new perspective on the role of PtdIns 3-kinase in plant cells: their results show that PtdIns 3P is formed in plant nuclei and that a class III PtdIns 3-kinase is localized at active nuclear transcription sites. The authors first demonstrate that both PtdIns 3P and PtdIns 4P are present in isolated soybean nuclei, and the identity of the lipids is confirmed by high performance liquid chromatography of deacylated lipid headgroups. When nuclei were isolated in the presence of detergent, the amount of PtdIns 4P in the nuclei was reduced, whereas the amount of PtdIns 3P was unchanged. From these data, the authors conclude that PtdIns 4-kinase activity is associated with the nuclear envelope, whereas PtdIns 3-kinase activity and PtdIns 3P reside within the nuclear matrix. In order to study the nuclear localization of PtdIns 3-kinase in more detail, a monoclonal antibody was generated against soybean PtdIns 3-kinase and used for immunolocalization studies. Immunolabeling of PtdIns 3-kinase was observed mainly in the nucleolus. Root sections were probed with both anti-PtdIns 3-kinase and anti-Br-UTP antibodies, after in vitro transcription in the presence of Br-UTP. Colocalization of the PtdIns 3-kinase and Br-UTP signals suggests that PtdIns 3-kinase is associated with active nuclear or nucleolar transcription sites. From these data, a new function is implied for plant PtdIns 3-kinase and/or PtdIns 3P in transcriptional regulation, in addition to previously established functions in vesicle trafficking.

Although the functional significance of a nuclear phosphoinositide cycle is not well understood, it is becoming increasingly clear that in animals and yeast many of the phosphoinositides and the corresponding lipid kinases are present in the nucleus (Divecha et al., 2000). Both type I and type II PtdInsP kinases have been shown to be associated with distinct subnuclear domains, known as nuclear speckles, which are functionally linked to mRNA metabolism and, therefore, may play a role in mRNA splicing (Boronenkov et al., 1998). A role for phosphoinositides in regulating nuclear transcription is also implicated by the specific binding of PtdIns(4,5)$P_2$ to histones H1 and H3 (Yu et al., 1998). York et al. (1999) have shown that inositol hexakisphosphate ($InsP_6$) plays an essential role in the export of mRNA from the nucleus and that a kinase with dual specificity for Ins(1,4,5)$P_3$ and Ins(1,4,5,6)$P_4$ is a vital part of a complex of transcription factors directing the expression of genes involved in arginine metabolism (Odom et al., 2000).

Some evidence exists from the animal literature that the class I, and potentially class II, PtdIns 3-kinases are present in the nucleus (Maraldi et al.,1999) and that they may translocate to the nucleus in response to stimulation (Bavelloni et al., 1999). However, no data have been reported so far in any eukaryotic system of a class III PtdIns 3-kinase in the nucleus. The information provided by Bunney et al. adds another piece to the puzzle that is the complex regulatory network of phosphoinositides and thereby opens new avenues for investigation. For example, it will be interesting to see whether translocation of a class III PtdIns 3-kinase to the nucleus plays a role in regulating transcription in response to a stimulus in plants. Because of the relative simplicity of plant PtdIns-3P metabolism, studies of the plant nuclear PtdIns 3-kinases should continue to provide insights that will

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help to delineate the functional roles of nuclear phosphoinositides.

The results presented by Bunney et al. are intriguing also from an evolutionary standpoint because the class III PtdIns 3-kinases are found throughout the eukaryotic realm in mammals, insects, slime molds, yeast, algae, and plants and may represent the primordial form of PtdIns 3-kinases. The ubiquitous nature of the class III PtdIns 3-kinases and their nuclear localization may reflect functional evolution of PtdIns 3P from the nucleus to membrane vesicles and ultimately the plasma membrane. In this regard, it will be important to determine whether a nuclear role for the enigmatic PtdIns 3P is unique to plants or whether the findings by Bunney et al. will be corroborated in other eukaryotic systems. In any event, in a field in which plant science is frequently modeled after animal research, it is inspiring when a new paradigm is proposed based on novel plant phosphoinositide research.

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MEETING REPORT

# Cell Biology of Plant and Fungal Tip Growth—Getting to the Point

Tip growth is a process that has many similarities in diverse walled cells such as pollen tubes, root hairs, and hyphae. However, due to the diversity of the experimental systems, it is unusual for those working on the phenomenon to have the opportunity to get together and compare systems and concepts. From June 19 to 23, 2000, NATO, the European Commission and the Università di Siena sponsored a NATO Advanced Research Workshop which brought together 75 of the current tip growth investigators for a focused exchange of information and ideas on this fascinating topic. Much of the work will appear in a dedicated volume (Geitmann et al., 2000), and abstracts of the presentations can be viewed at: http://www.dpw.wageningen-ur.nl/epc/NATO-tipgrowth. The aim of this report is to identify trends in the field and bring together an introduction to a literature that is often widely dispersed.

## ACTIN, NOT MICROTUBULES, DOES MUCH OF THE JOB

A clear consensus among meeting participants, consistent with a recent review (Geitmann and Emons, 2000), was that an apical concentration of actin exists in all cell types examined. However, the organization of apical actin was not conserved, varying from diffuse fine filaments permeating the apical cytoplasm to coarser filaments, large patches, and smaller plaques, all predominantly associated with the apical plasma membrane. Apical actin was suggested to regulate exocytosis directly (Irene Lichtscheidl, University of Vienna, Austria), to set up subapical cables involved with organelle transport (Peter Hepler and Luis Vidali, University of Massachusetts, USA), and to be responsible for the regulation of tip extensibility (I. Brent Heath, York University, Canada). Supporting the latter were observations of tip swelling or deformation following actin disruption in many diverse cells (Frantisek Baluška, University of Bonn, Germany; Norbert de Ruijter, Wageningen University, The Netherlands; I.B. Heath; Tijs Ketelaar, Wageningen University, The Netherlands; L. Vidali). However, Orest Demkiv (Institute of Ecology of the Carpathians, Ukraine) showed an unexplained contrary result in moss cells, suggesting intercellular variability.

Alternative functions of actin included organizing morphogenically important accumulations of endoplasmic reticulum (Brigitte Buchen, University of Bonn, Germany) and orienting cell wall fibrils (Christos Katsaros, University of Athens, Greece) in algae, and positioning the translation apparatus in yeasts (Alison Adams, University of Arizona, USA; Jiri Hašek, Czech Academy of Sciences, Czech Republic). The latter is likely to be even more important in longer and highly polarized cells, such as pollen tubes and fungal hyphae.

The repeated demonstrations of differential organization of apical actin, relative to subapical regions, and of its configurational changes upon exposure to external signals (N. de Ruijter; A. Geitmann, Wageningen University, The Netherlands) introduced the whole question of actin regulation. A number of actin binding proteins with physiologically relevant properties were localized to the appropriate regions of cells. These include villin (P. Hepler), profilin (L. Vidali), ARP 2/3 (F. Baluška; David Drubin, University of California at Berkeley, USA; Whitney Hable, University of Utah, USA) and ABP1 (D. Drubin). However, it is clear that this list is only just started. Hepler made a very compelling case for the integration of actin, actin binding proteins, and $Ca^{2+}$ gradients in the organization of the apical cytoskeleton.

The factors instrumental in initiating and controlling the assembly of the tip growth apparatus were investigated in developing algal zygotes (D. Kropf, University of Utah, USA; W. Hable), yeast buds (D. Drubin) and hyphal branches (I.B. Heath; Sandra Jackson, University of Christchurch, New Zealand). In each system, assembly of characteristic actin arrays adjacent to the plasma membrane was one of the earliest events described. Both Hable and Drubin were able to add actin-related proteins (ARP2/3) as participants in this ensemble. The factors that precede the assembly of the actin arrays remain elusive. Heath presented evidence that a pulse of $[Ca^{2+}]_{cyt}$ was part of the early signaling system, but Jackson was unable to detect such pulses and presented evidence from the characteristics of micropipette-applied inducers that $Ca^{2+}$ were not initiators.

In contrast to abundant evidence for a critical role for actin in regulating tip growth, it was repeatedly emphasized that microtubules have only an indirect role. Polarized tubular growth could be initiated or maintained in the absence of microtubules in diverse cells (F. Baluška; Giampiero Cai, University of Siena, Italy; D. Kropf). However, normal steering (see “Steering and Navigating”)

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does involve microtubules in as yet undetermined ways.

### "SPECTRIN" CONTRIBUTES TOO

In at least two phylogenetically very different organisms, *Neurospora* hyphae and *Chara* rhizoids and protonemata, proteins with substantial similarities to animal spectrins contribute to tip growth. In *Neurospora*, they form a cap concentrated at the growing plasma membrane (I.B. Heath) whereas in *Chara*, conspicuously and surprisingly, they are not associated with the plasma membrane but instead concentrate in the apical accumulation of endoplasmic reticulum, which plays a vital role in gravitropism (B. Buchen). These proteins are primarily identified by their size and their reaction with antibodies and have previously been reported in other hyphal and root hair tips (Kaminskyj and Heath, 1995; de Ruijter et al., 1998). Their precise relationship to animal spectrins remains to be seen, but their location and currently known similarities make them very interesting to the tip growth process.

### ION REGULATION

All tip-growing cells that have been examined generate a tip-high gradient of cytoplasmic [$Ca^{2+}$], which apparently is obligatorily involved in active growth (Malhó et al., 1994; Holdaway-Clarke et al., 1997; Hyde and Heath, 1997; Wymer et al., 1997). This observation was reinforced throughout the meeting (José Feijó, Instituto Gulbenkian Ciencia, Portugal; I.B. Heath; P. Hepler; S. Jackson; Rui Malhó, University of Lisbon, Portugal; Kenneth Robinson, Purdue University, USA). A similar gradient of cytoplasmic [$H^+$] has also been reported in some cells (Feijó et al., 1999; J. Feijó; K. Robinson). Exciting aspects of the ion studies were both the increased precision of correlation between the concentrations in the gradients, ion fluxes measured with vibrating probes and oscillating growth rates (see "Subtle Jackhammers"), and the addition of other ion fluxes to the equation. For example, Robinson showed that $[Ca^{2+}]_{cyt}$ resulting from apical $Ca^{2+}$ influx follows a growth pulse, leading to a model in which extension results in elevated $[Ca^{2+}]_{cyt}$ and stimulated exocytosis, producing the necessary membrane and wall material for the next growth pulse. The $Ca^{2+}$ stimulation of exocytosis probably also relates to regulation of both vesicle transport and the properties of the actin cytoskeleton, indicating the multi-functional roles of $Ca^{2+}$, as emphasized by Hepler. The $Ca^{2+}$ influx is followed by $H^+$ and $K^+$ influxes, the significance of which is unclear. Feijó noted that $K^+$ fluxes tended to be highly variable in magnitude and direction, suggesting that they simply balance other ions. Nevertheless, Polydefkis Hatzopoulos (Agricultural University of Athens, Greece) showed that $K^+$ transport is critical for root hair growth (but, surprisingly, apparently not pollen tubes) in Arabidopsis.

It generally has been assumed, and demonstrated, that the tip-high gradient of $[Ca^{2+}]_{cyt}$ is generated and maintained by $Ca^{2+}$ influx through the apical plasma membrane, but this is not always so (Lew, 1999), leading to the suggestion of some form of internal recycling, or "bootstrapping" system (Jackson and Heath, 1993). Sara Torralba (York University, Canada) presented the first evidence for such a system by showing $Ca^{2+}$ concentrated in the secretory vesicles in *Neurospora* hyphal tips, adding a new level of complexity to the ion regulatory systems in tip growth.

While most attention has previously focused on cations, it is emerging that anions are also important. Both Feijó and Laura Zonia (Institute of Experimental Botany, Czech Republic) showed pulsed apical efflux of $Cl^-$ related to pulsed growth of pollen tubes and concluded that this efflux is important to water fluxes and thus turgor regulation. The other anions introduced were reactive oxygen species. Robinson showed that $O_2$ consumption at pollen tube tips increases following a growth pulse, but apparently not as a supplier of metabolic energy. He suggested that apical superoxide is converted to $H_2O_2$, which functions to regulate pectin cross-linking and thus cell wall extensibility, a suggestion supported by his induction of tip swelling with the antioxidant ascorbate.

### KINASES AND G-PROTEINS

Given the ubiquity of signaling systems based on kinases and G-proteins, it is not surprising that there was substantial evidence for their involvement in diverse tip-growth systems. Baluška showed the accumulation of MAP kinases at root hair initiation sites, Malhó found calcium kinases at the plasma membrane and in the cytoplasm in tips of pollen tubes, and Drubin showed the importance of two other kinases in yeast bud cytoskeleton organization. The latter seem to be most important for endocytosis. The most direct evidence for kinase function in tip morphogenesis came from the severe morphological distortions that John Esseling (Wageningen University, The Netherlands) induced by antisense inhibition of several serine/threonine kinases in Arabidopsis root hairs.

Assorted G-proteins are specifically expressed and/or localized in pollen tubes (Victor Žárský, Institute of Experimental Botany, Czech Republic), yeast buds (D. Drubin) and mating fungal hyphal tips (Marjatta Raudaskoski, University of Helsinki, Finland).

In none of the systems has it been possible to integrate the kinase and G-protein data with the ion and cyto-

skeletal data to produce a clear and coherent story, although certainly the yeast bud system is fast approaching that state.

## HOW THE MEMBRANES COME AND GO

Because tip growing systems entail so much exocytotic activity, it is important that they accurately orchestrate both the production and placement of their membrane producing systems. Previous observations (Miller et al., 1995; Moscatelli et al., 1995; Steinberg, 1998) have described a number of molecular motors that are involved in vesicle and organelle transport to the growing tips. However, additional complexities emerged. For example, Geoff Hyde (University of New South Wales, Australia) showed that in response to perturbations, the distributions of both endoplasmic reticulum and a tubular vacuolar system of hyphae were altered in specific ways, indicative of positioning systems for these membrane components. The basis for these rearrangements was not addressed, but in *Chara* cells a different, but highly organized, apical aggregation of endoplasmic reticulum was maintained with the involvement of a spectrin-like protein (B. Buchen).

At a later step in the exocytotic pathway, both Rosa-Maria Lopez-Franco (Instituto Technologico de Estudios Superiores de Monterrey, Mexico) and Salomon Bartnicki-Garcia (University of California at Riverside, USA) demonstrated the behavior of a highly concentrated apical aggregation of vesicles and actin (Bourett and Howard, 1991) known as a Spitzenkörper. Spitzenkörpers represent an unexplained stage in the direction of the vesicles to the apical plasma membrane. SNARE proteins assist in ensuring correct vesicle fusions during exocytosis, and for the first time in a tip-growing system, Gagan Gupta (York University, Canada) showed a tip-high gradient of SNAREs in hyphae of *Neurospora*.

Another feature of membrane behavior that attracted considerable attention was retrieval by endocytosis. Both Lichtscheidl and Drubin showed evidence for fluid phase endocytosis (presumably with concomitant membrane internalization) in diverse cells, apparently generated by plasma membrane–associated actin. In yeast, Drubin also showed that there are clathrin-interacting proteins involved. Nick Read (University of Edinburgh, Scotland) presented impressive images of the time course of internalization of a membrane-specific dye in fungal hyphae and showed evidence for it passing through a membrane recycling system to become exocytosed back to the tip. However, in his data there was no direct evidence for endocytosis of membrane segments, as opposed to excision, internalization, and recycling of individual dye molecules, an equally plausible and interesting interpretation.

## STEERING AND NAVIGATING

Tip growth is a mechanism for exploring the environment and getting cellular constituents to specific locations. It follows that cells need to be able to detect environmental cues (See "Environmental Sensing") and steer accordingly. Both algal zygotes and fungal hyphae retain a sense of direction. Kropf showed that unless other stimuli were applied, algal zygotes remembered the site of sperm entry and used this as the cue for tip growth initiation. In the same system, Sherryl Bisgrove (University of Utah, USA) showed that both microtubules and actin interact in "remembering" and responding to this site. Likewise, Lopez-Franco showed that laser tweezer positioning of the hyphal Spitzenkörper could steer hyphal growth, but only up to a point. Attempts to force hyphae to grow backward failed; they clearly "knew" which way they should go and were not going to be deviated too far from the straight and narrow! The mechanisms were not investigated but could involve microtubules interacting with actin, as in the algal zygotes, because hyphae steer poorly following microtubule disruption (Riquelme et al., 1998). Demkiv also showed that moss protonemata autotropism is guided by microtubules.

Perhaps the most dramatic demonstration of steering, or the lack thereof, was Hartmut Quader's (Universtät Hamburg, Germany) demonstration of helical growth in pollen tubes treated with methylxanthines and cyclopiazonic acid. Both microtubules and actin filaments were implicated in its regulation, although loss of microtubules did not suppress it. Perhaps the most fascinating but unexplained aspect of this and previous demonstrations of helical tip growth is that they are evidence for a rotary motor in the tips.

Given the indications of cytoskeletal function in navigation, it is perhaps no surprise that Malhó (Malhó et al., 1994) was able to show that $[Ca^{2+}]_{cyt}$ is also part of the steering system in pollen tubes.

## SUBTLE JACKHAMMERS AND TURGOR REGULATION

Pulsatile growth, with concomitant oscillations in ion fluxes and $[Ca^{2+}]_{cyt}$, is a well-established phenomenon in pollen tubes (Pierson et al., 1995; Geitmann and Cresti, 1997; P. Hepler; K. Robinson), although it emerged at the meeting that such is not always the case. There are variations in this behavior between species and with age in a single species. Pulsed growth is also reported in hyphae (Lopez-Franco et al., 1994), but Jackson showed that such can arise from technical artifacts and that some hyphae lack pulses.

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A number of people suggested that the pulses are primarily generated by local oscillations/changes of turgor pressure (J. Feijó; K. Robinson; L. Zonia) and may be part of an oscillatory feedback mechanism (with regular frequency) that includes turgor, secretion, wall plasticity, and ions all as regulatory elements. However, Nicholas Money (Miami University, USA) showed that some hyphae actually grow faster with "zero" (or at least very low) turgor pressure, making the important point that turgor is not essential for the process of tip growth itself. Nevertheless, he did point out that normal turgor is essential for hyphal penetration of solid substrates, thus indicating that the analogy between pulsatile growth and a jackhammer may not be far fetched!

### ENVIRONMENTAL SENSING

Tip-growing cells need to communicate in complex ways with their environment, both to receive and to send signals. Examples of this are plant pathogenic fungi, in which Harvey Hoch (Cornell University, USA) showed the ability both to sense a correct (e.g., leaf) surface before they adhere and germinate and to locate stomata with minimal directed tip growth. Hyphae and spores sense micrometer-sized surface features and respond either by secreting adhesives that will attach to hydrophobic surfaces (including Teflon) or by steering their growing tips. The mechanisms are unclear but involve both the cytoskeleton and $Ca^{2+}$. A fascinating aspect of this sensing is that it traverses the cell wall to the cytoplasm, a process likely to involve plasma membrane to cell wall linkages. Such have been shown to be mediated by RGD-recognizing integrin-like proteins (Corrêa et al., 1996). Ashley Garrill (University of Christchurch, New Zealand) showed evidence for similar adhesions in non-sensing hyphae, suggesting that they are widespread.

One of the most critical sensing systems involving tip-growing cells is that mediating pollen–stigma interactions. In compatible interactions, the pollen tubes must establish and retain appropriate adhesions with cells of the transmitting tissues. Both Patricia Bedinger (Colorado State University, USA) and Elizabeth Lord (University of California at Riverside, USA) described secreted proteins likely to be involved in these interactions. Bedinger described an extensin-like pollen tube wall protein, disruption of which leads to irregular spiral tube growth in styles, and Lord described a small protein and a polygalacturonan, both secreted by pollen tubes, which combine to mediate adhesion of the tube to stylar tissues. Incompatible interactions entail the exchange of signaling molecules between pollen and stigma, the result of which is the failure of pollen tube growth. Both Anna Kalinina (St. Petersburg State University, Russia) and Geitmann presented very interesting indicators of what these molecules may be. Kalinina was able to show that the incompatibility reactions of rye plants could be altered with $Ca^{2+}$ channel blockers. Geitmann was able to extend a comparable system to the cytoskeleton by showing major changes in actin organization in poppy pollen exposed to an incompatibility (S locus) protein. Most interestingly, these changes did not mirror those elicited by actin-depolymerizing drugs. They are to some degree similar to cytoskeletal changes in apoptotic cells, introducing the exciting idea that incompatibility may represent another example of apoptotic cell death in plant biology.

### WHERE TO NEXT?

In concluding remarks, Robinson summarized a number of outstanding questions that emerged from the meeting. These include: 1) greater effort to understand the similarities and differences between species and cell types; 2) more focus on in situ pollen studies to determine the validity of the currently emerging consensus from in vitro studies; 3) improved preservation methods to better understand the critical organization of the players in all systems; 4) more emphasis on the mechanisms of two-way communication across the apical plasma membrane; 5) greater effort on understanding the localized properties and changes in the apical cell wall, and interactions with cytoplasmic turgor pressure; 6) focus on directionality memory mechanisms that determine the direction of growth of tubular cells, and 7) identification and analysis of mechanosensitive channels in pollen tubes and other cells where they have yet to be described. No doubt the answers to some of these questions will make a solid base for a future tip growth meeting, hopefully in less than the 10 years since the last multidisciplinary work on the topic (Heath, 1990). Ensuring that the location for such a meeting matches the ambiance of Siena remains a challenge for the next organizers!

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LETTER TO THE EDITOR

# ER Retention of Soluble Proteins: Retrieval, Retention, or Both?

Pagny et al. (2000) have recently published an article that touches on a very controversial and important process in the secretory pathway, the export of soluble proteins from the endoplasmic reticulum (ER). The results that are reported confirm data reported previously (Pedrazzini et al., 1997; Crofts et al., 1999; Frigerio et al., 1999); however, the authors reach an opposite conclusion. It therefore appears important and timely to initiate a discussion about this topic.

Without any doubt, the issue of ER export is far from settled and discussions have been ongoing for almost a decade (Armstrong, 1995). Two models are currently competing for general acceptance. One model describes ER export as a non-selective diffusion into anterograde ER-derived transport vesicles (bulk-flow). The second model postulates that proteins are actively selected and enriched during ER export (active transport). The bulk-flow model is supported by experiments in vivo using mammalian cells (Wiedman et al., 1984, 1987) and plant cells (Denecke et al., 1990; Hunt and Chrispeels, 1991). The active transport model arose from studies using in vitro systems; the strongest evidence emerging from the fact that purified ER-derived COPII vesicles

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were enriched for yeast α-factor, a secretory cargo molecule, whereas the ER resident chaperone BiP was absent in these vesicles (Barlowe et al., 1994). Because of the strong evidence for both models, the possibility that bulk-flow and active selection operate in the same transport system, perhaps through different types of transport vesicles, should not be excluded (Vitale and Denecke, 1999).

Pagny and co-workers have now shown that the ER resident protein calreticulin does not carry complex glycans even under conditions of ER stress in the floury 2 mutant of maize. This confirms results obtained with ER-retained assembly defective phaseolin (Pedrazzini et al., 1997), KDEL-tagged phaseolin (Frigerio et al., 1999), as well as results on calreticulin under normal physiological conditions or during overproduction of this protein and subsequent saturation of the ER retention machinery (Crofts et al., 1999). It is not known whether assembly defective phaseolin is capable of leaving the ER or whether it is recycled from the Golgi via association with BiP, but either model must incorporate the fact that the protein does not acquire complex glycan modifications. However, the rapid export of phaseolin from the ER is well established (Frigerio et al., 1998), and ER export of calreticulin was achieved via overexpression (Crofts et al., 1999). In these cases, retrieval and subsequent accumulation of complex glycan containing forms in the endoplasmic reticulum could not be detected (Crofts et al., 1999; Frigerio et al., 1999). The conclusion from these reports is that retrieval of HDEL proteins that escape from the ER occurs from a Golgi compartment devoid of glycan-processing enzymes, such as the cis-most and the *cis*-Golgi cisternae.

There is good evidence that calreticulin leaves the ER frequently. First, deletion of the HDEL motif causes secretion, which demonstrates its dependence on the HDEL motif to remain in the cells (Crofts et al., 1999). The retrieval of HDEL proteins from the Golgi complex is well established in yeast and mammalian cells, and the functional complementation of AtERD2 in yeast certainly demonstrates that the plant HDEL receptor functions in the same way (Lee et al., 1993). Second, truncated calreticulin accumulates in transgenic plants to much lower levels than wild-type calreticulin when overexpressed using a strong viral promoter (Crofts et al., 1999). The discrepancy between truncated, and wild-type calreticulin was approximately a factor of 100 between the best overproducing plants. This is likely due to degradation of the truncated molecule in a post-ER compartment after ER export, thus under-representing the amount secreted in the absence of HDEL.

Pagny and co-workers claimed that calreticulin does not leave the ER and that HDEL-mediated retrieval of this ER resident is so minor that it would be undetectable via biochemical analysis. This claim is contradictory to our own interpretations and is deduced indirectly from findings with the HDEL-tagged secretory protein invertase (InvFlagHisHDEL). The authors argue that for this cargo molecule, HDEL-mediated retrieval occurs also from post-*cis*-Golgi cisternae, and that complex glycan containing invertase is frequently recycled back to the ER. In our opinion, the authors have not presented evidence to support their claim. Pulse–chase experiments were conducted and subsequent cellular extracts (the authors refer to "intracellular medium") were obtained through homogenizing entire cells and subsequent centrifugation of debris. The supernatant will contain Golgi vesicles and transport vesicles in addition to ER, as well as proteins trapped within the cell walls. InvFlagHisHDEL carrying complex glycans may therefore be in transit through the Golgi apparatus or simply in the cell walls. Also, it appears strange that microsomes contained undetectable amounts of secretory InvFlagHis and the authors refer in the text to "exclusive detection in the medium" (Figure 3, Pagny et al., 2000), whereas, "intracellular medium" contained easily detectable amounts in Figure 6 (Pagny et al., 2000). The two figures contradict each other, particularly because InvFlagHis and InvFlagHisHDEL in the "intracellular medium" are suddenly equal in abundance (Figure 6).

Until it has not been excluded by direct experimental analysis that InvFlagHisHDEL containing complex glycans are not derived from the cell walls in Figure 6, but in fact are localized in the ER, it is premature to suggest that recycling of complex modified glycoproteins back to the ER occurs at all. Such analysis could be done simply by repeating the pulse–chase experiment with washed protoplasts prepared from the BY2 cells. But since a protoplast extract would also contain Golgi membranes and transport vesicles, proof for recycling to the ER would be the purification of ER membranes and demonstration of a magnesium shift in sucrose gradients of complex modified InvFlagHisHDEL. This would show that complex InvFlagHisHDEL has reached the ER.

We argue that even if InvFlagHisHDEL does indeed recycle from the *trans*Golgi back to the ER, this does not mean that ER export of calreticulin is marginal just because it does not recycle from the *trans*Golgi. Perhaps calreticulin is degraded when it reaches a post-*cis*-Golgi compartment whereas invertase is more stable? It is also possible that the HDEL motif of calreticulin is better presented in its natural context and thus confers more complete recycling from the *cis*-Golgi apparatus. Perhaps this is not the case for InvFlagHisHDEL, causing escape to more distal regions of the Golgi, a suggestion that can also be derived from its partial

secretion. It has already been shown that tagging with ER retention motifs is not always sufficient to obtain efficient retention. KDEL-tagged phytohemagglutinin was only partially retained in the ER and the nuclear envelope, the majority still reached the vacuoles (Herman et al., 1990). Yet a significant proportion of the recombinant protein was now endo-H sensitive, which would correspond well with the model in which retrieval of ER residents occurs mainly from the *cis*-Golgi apparatus.

Clearly, further work is needed to clarify the issue about ER export and retention of soluble proteins. This point is very important, as it also relates to the possible mechanism of quality control and ER retention of malfolded proteins. Do malfolded proteins leave the ER and recycle via association with ER chaperones such as BiP, or are they excluded from ER export? A constructive discussion and renewed research will certainly provide answers to these questions.

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# Reply: Glycobiology and the Plant Cell—A World of Information

Releasing appropriate information to members of the scientific community as well as re-emphasizing that glycobiology-related events are information-based processes call for our detailed answer to the letter by Pimpl and Denecke.

Indeed, the rapidly increasing amount of information now available on N-glycan maturation permits quick progress in the characterization of mechanisms that govern protein targeting along the plant secretory pathway. Our approach is based on carrot cell wall invertase (Inv) as a reporter glycoprotein fused

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with an endoplasmic reticulum (ER) retention signal (HDEL), a purification (His) tag, and an immunodetection (Flag) tag. This InvFlagHisHDEL was used as bait to collect information on N-glycan structures when a protein is retained in the ER exclusively on the basis of the HDEL-dependent recycling machinery. Actually, a major hurdle in this project was the selection of our reporter glycoprotein that, according to our specifications, had to be extracellular and stable, and that had to undergo N-glycan maturation during its transport through the Golgi apparatus. We also needed to prevent contamination of the ER resident reporter protein fused with HDEL by secreted forms of the same reporter that would escape the ER retention machinery. The carrot cell wall invertase appeared to be a perfect model, particularly because of the established cleavage of its C-terminal propeptide that naturally occurs during transport to the cell wall. Accordingly, we fused our immunopurification and immunodetection tags in a C-terminal position from this cleavable propeptide so that the minor amounts ($<$10%) of InvFlagHisHDEL that would escape the ER retention machinery to be secreted would have lost their tags and could not contaminate the products immunopurified from a microsomal fraction with antibodies specific for the Flag epitope. In a pulse–chase experiment, we have shown that the whole InvFlagHisHDEL retained in the ER progressively acquires EndoH and PNGase F resistance. According to what we currently know about the specificity of these endoglycosidases, this result indicates that InvFlagHisHDEL N-glycans mature and, notably, are $\alpha$–1,3-fucosylated during the signal-mediated recycling of the reporter from the Golgi apparatus (Pagny et al., 2000). Hence, retrieval of the whole ER resident invertase occurs down to the medial and *trans* Golgi. Due to this precise strategy and a glycan analysis of this "artificial reticuloplasmin," we have obtained strong evidence for a very active retrograde pathway between the Golgi and ER in plants.

Pimpl and Denecke also comment on our analysis of the natural ER resident protein calreticulin, and they compare our results (Pagny et al., 2000) to theirs (Crofts et al., 1999). In Figure 1 of their paper, traces of truncated calreticulin deleted from its HDEL C-terminal motif (calreticulinΔHDEL) are barely detectable in the incubation medium of tobacco protoplasts. Anyone who has cultured tobacco protoplasts over an extended incubation time (24 hr in Crofts et al., 1999) has observed that many protoplasts burst during incubation and, consequently, release their intracellular content, including ER proteins, vacuolar proteases, and the like into the incubation medium. Needless to say, this protease-rich medium is the perfect environment for uncontrolled proteolytic maturation of intracellular proteins released by protoplasts that have burst open. Under these conditions, we find highly questionable the authors' claims that (1) trace amounts of calreticulinΔHDEL found in the protoplast medium have been secreted and (2) the lower molecular weight of calreticulinΔHDEL found in this medium indicates that glycan processing has taken place during its transport from the ER to the cell surface (Crofts et al., 1999). Even if one admitted that the trace amounts of both BiPΔHDEL and calreticulinΔHDEL found in the protoplast incubation media (Crofts et al., 1999) do represent a fraction that is secreted after HDEL deletion and that would normally recycle if the HDEL signal were present, these results would strongly reinforce our conclusions, namely that ER retention of these proteins relies primarily on mechanisms other than HDEL-mediated recycling.

Surprisingly, Pimpl and Denecke consider that our results and their results are similar, whereas both teams have reached opposite conclusions. The results are not similar. In one case, Crofts et al. (1999), the calreticulin N-glycan structure is so roughly defined with a low specificity endoglycosidase, that one cannot determine whether calreticulin recycles from the Golgi back to the ER. In the other case, the wide variety of tools now used by plant glycobiologists, such as glycan-specific antibody probes, lectins, highly specific endoglycosidases, and mass spectrometry (Bardor et al., 1999), has helped to determine that calreticulin harbors Man8GlcNAc2 and Man9GlcNAc2 N-glycans**.** The latter are competent for modification by Golgi enzymes and, hence, their structure does not support a recycling of calreticulin through the Golgi, not even through the early *cis* Golgi compartment where $\alpha$-1,2-mannosidase I is localized (Nebenführ et al., 1999).

Our results and those of Pimpl and Denecke are not similar unless one considers that stably transformed suspension-cultured tobacco cells, which have been studied after several months of subculturing after transformation (Pagny et al., 2000), will provide the same type of information about the secretory pathway as do protoplasts overexpressing a reporter protein at such a high level that ER cisternae and nuclear envelopes are dilated (Crofts et al., 1999). What could one seriously conclude about the normal transit downstream of such a heavily constipated ER? At best, that protein secretion occurs via a default pathway (i.e., a conclusion that is strongly supported by results obtained during the last century [Denecke et al., 1990; Hunt and Chrispeels, 1991]).

As illustrated in Pagny et al. (2000), plant glycobiologists have the tools necessary to demonstrate that calreticulin is mainly retained in the ER by mechanisms that rely primarily on signals other than the HDEL motif. The C-terminal extension of this plant reticuloplasmin likely remains a "security signal" that, at least under physio-

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logical conditions, very efficiently prevents the chaperone from being secreted, should it ever escape the ER. Our continued investigations will help us to characterize the mechanism(s) and signal(s) that strongly limit calreticulin diffusion in vesicles exported from the ER.

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