FEBS Letters 518 (2002) 88–92 FEBS 26040

Effects of brefeldin A and nordihydroguaiaretic acid on endomembrane dynamics and lipid synthesis in plant cells

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Received 21 January 2002; revised 25 March 2002; accepted 25 March 2002

First published online 10 April 2002

Edited by Ulf-Ingo Flügge

Abstract Effects of brefeldin A (BFA) and nordihydroguaiaretic acid (NDGA) on endomembrane structures and lipid synthesis were compared in maize root cells and tobacco Bright Yellow-2 cells. Immunofluorescence and electron microscopy studies showed that NDGA altered the structure and distribution of the endoplasmic reticulum (ER) within 1 h but not of the Golgi apparatus whereas, as shown previously, BFA altered that organization of the Golgi apparatus and, only subsequently, of the ER. Biochemical studies revealed that both drugs and especially BFA led to a strong inhibition of the phytosterol biosynthetic pathway: BFA led to accumulation of sterol precursors. The importance of phytosterols in membrane architecture and membrane trafficking is discussed.

Key words: Endoplasmic reticulum; Golgi dynamics; Brefeldin A; Nordihydroguaiaretic acid; Sterol

1. Introduction

In many cases, plant cells show a different sensitivity to Golgi transport inhibitors than that found in mammalian cells [1]. The exact nature of these discrepancies is not understood. For instance, the popular Golgi-disrupting drug brefeldin A (BFA) has distinctive effects in plants and animals [1–3]. Plant genomics indicate however that plant cells possess the same elements of a putative protein transport machinery as mammalian or yeast cells [4,5]. Distinctive endomembrane lipid composition [6,7] and metabolism may explain differences of endomembrane machinery behavior after inhibitor treatment. Moreover, it is known from animal studies that BFA may have profound effects on lipid metabolism [8,9].

This study aimed to compare the effects of two Golgi transport inhibitors on endomembrane structures and lipid biosynthesis: BFA, which is the only drug reported so far able to induce a complete and reversible disruption of the plant Golgi stack in a plant specific manner [1,10], and nordihydroguaiaretic acid (NDGA), whose effects on plant endomembranes have never been reported. In animal cells, the target of BFA is the machinery involved in budding of COPI vesicles from the Golgi apparatus [11]. The molecular basis of BFA effects in animal cells and yeast is the inhibition of guanine nucleotide exchange on ADP ribosylation factor (ARF) proteins catalyzed by proteins having a SEC 7 domain [12,13]. In plant cells, the molecular target(s) of BFA is (are) still unknown, although coat protein recruitment has been shown to be inhibited by BFA in vitro [4].

NDGA, an inhibitor of lipoxygenases and cyclooxygenases, has also been reported to inhibit mammalian vesicle-mediated protein transport [14], and its effects on Golgi morphology were similar to the ones described with BFA as it may induce the redistribution of Golgi proteins into the endoplasmic reticulum (ER) albeit by a COPI-independent mechanism [15–17].

To analyze NDGA effects on the endomembrane dynamics of plant cells, immunocytochemical studies of ER and Golgi apparatus in root tissues and cell cultures were carried out using confocal laser scanning microscopy. Results were compared with the known BFA effects on these structures [10,18]. Furthermore, the impact of both NDGA and BFA on lipid biosynthesis was assessed by biochemical studies.

2. Materials and methods

2.1. Plant material

Nicotiana tabacum Bright Yellow-2 (BY-2) suspension cultured cells were grown in a modified Murashige and Skoog medium as described [18]. Maize caryopses (*Zea mays*, LG31, Limagrain, France) were immersed in tap water for 3 h and germinated in Petri dishes on moist filter paper in the dark at 26°C. Root apices were excised from 3-day-old shoots.

2.2. Drug treatments

BFA (Alexis Corp.) solutions were made from a 20 mg/ml (70 mM) stock solution in DMSO. Roots and the cell suspension were treated with a 100 μg/ml (350 μM) solution for 1 h as described [10,18].

A fresh stock solution of NDGA (Sigma) at 100 mM was prepared in DMSO. NDGA was used in a concentration range from 10 to 500 μM.

Controls were made with cells treated by DMSO at the highest concentration.

2.3. Immunofluorescence, electron microscopy and cytometry

Procedures were performed as described [18,19]. A rat monoclonal antibody JIM84 was used as a Golgi marker [20], and a mouse monoclonal antibody 2E7 was used as an ER marker [21]. Slides were observed either with a Reichert Polyvar fluorescence microscope or a Sarastro 2000 confocal microscope (Molecular Dynamics).

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0014-5793/02/$22.00
PII: S0014-5793(02)02651-0

For ultrastructural studies, specimens were fixed, impregnated by zinc–iodine–osmium and embedded in Spurr resins as described [10]. Observations were made on a Philips CM10 and micrographs taken on Kodak films.

Cell cycle analyses were performed by flow cytometry [22].

2.4. Lipid synthesis and analyses

Maize root apices were incubated for 2 h with BFA or NDGAff concentrations ranging from 10 to 500 μM, and with 10 μCi of [1-^{14}C]acetate (54 Ci/mol, Amersham). Controls were treated with DMSO at the highest concentration used for the drugs.

Polar lipids were analyzed as reported [23]. Neutral lipids were isolated on HPTLC plates (Merck 60F254) developed with hexane: ethylether:acetic acid (90:15:2, v/v) to give: diacylglycerols (R_f 0.10), sterols (R_f 0.17), fatty alcohols (R_f 0.22), sterol precursors (R_f 0.25), free fatty acids (R_f 0.30) and triacylglycerols (R_f 0.55). Sterols correspond to Δ^5 sterols (sitosterol, isofucosterol, 24-methyl cholesterol, cholesterol and stigmasterol). Sterol precursors include mainly cyclopropylsterols such as 4α-methylsterols and 4,4-dimethylsterols. Radioactivities of polar and neutral lipids were determined using a phosphorimager (Molecular Dynamics SI, Pharmacia).

3. Results

3.1. NDGA affects plant growth

After 6 h treatment within a range of 25–500 μM NDGA, growth of maize shoots was decreased by 30% at 50 μM and by 80% at 250 μM. Moreover, necrosis appeared at 250 μM, and tissues were dead at 500 μM.

For cell suspensions, the packed cell volume was decreased by about 80% at 75 μM NDGA. A cell viability test (propidium iodide exclusion) and subsequent observations at the light microscope showed that all cells were still viable after 24 h of 75 μM treatment, and cell morphology was unchanged (same cell size, dense cytoplasm). This inhibition was reversed by a 24 h wash. The frequency of mitosis fell after NDGA treatment: for instance, the mitotic index dropped from 8.2% to 7.8% after 1 h treatment with 25 μM NDGA, and to 5% after 3 h; in the case of 1 h treatment with 75 μM NDGA, the mitotic index went down to 5.7%, and stayed blocked at this value for 3 h of treatment. After 9 h, the mitotic indexes appeared to increase again, as if the cell was able to recover from NDGA effects. Cell cycle analysis allowed a better understanding of NDGA effects on cell cycle: NDGA-treated cell cultures (25–75 μM treatment) became enriched in G1 phase (up 10%), especially after 6 h of NDGA treatment. These results strongly suggest that NDGA effects on growth may be linked to a partial blockage of dividing cells in G1 phase.

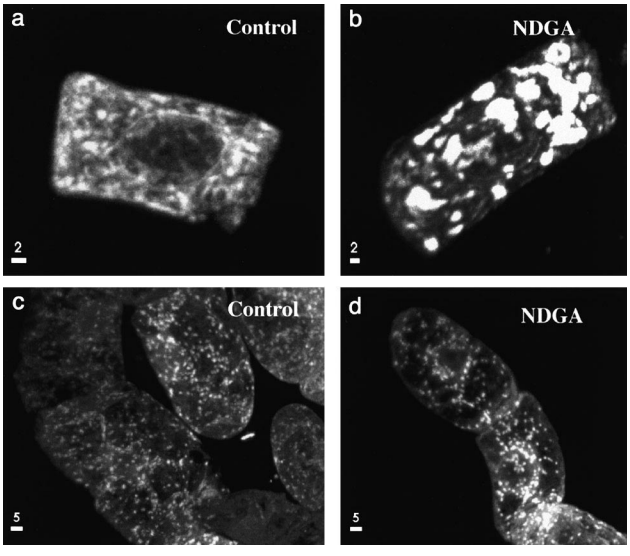

Fig. 1. NDGA effects on ER (a,b) and Golgi (c,d) in plant cells. a: Immunostaining pattern of ER with 2E7 antibody in isolated maize root cells. The ER appears like a tubular meshwork throughout the cytoplasm. The nuclear membrane in continuity with the ER is stained as well. b: NDGA effects (50 μM, 1 h): the ER clearly aggregates in numerous immunolabelled clusters, often attached to the nuclear membrane or plasma membrane. c: Immunostaining pattern of the Golgi with JIM84 antibody in BY-2 cells. Numerous Golgi units (stacks) appear dispersed throughout the cytoplasm. d: NDGA effects (50 μM, 1 h) on plant Golgi: the typical immunofluorescent pattern of BY-2 cells as revealed by JIM84 is not affected by the NDGA treatment.

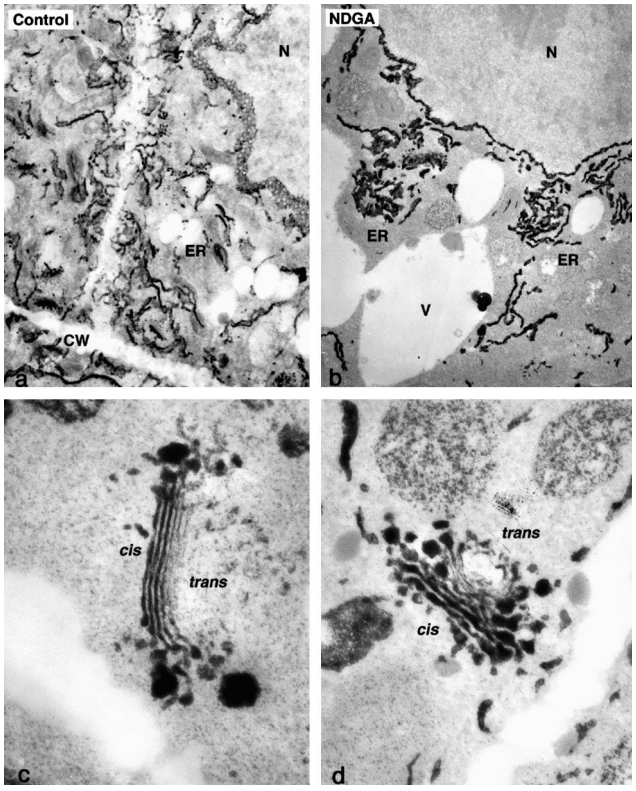

Fig. 2. Observations of the ER (a,b) and Golgi apparatus (c,d) on ultrathin sections of NDGA-treated maize root cells. a,c: Control cells; b,d: 75 μM NDGA-treated cells. a,b,: ×11 500; c,d: ×50 000; ER: endoplasmic reticulum; V: vacuole; CW: cell wall; N: nucleus.

3.2. NDGA alters the three-dimensional organization of the ER, but not the Golgi apparatus

When stained with 2E7 antibody, the ER of maize root cells appears like a network radiating from the nucleus throughout the whole cytoplasm (Fig. 1a). This normal pattern was disturbed when the shoots had been treated with 25–50 μM NDGA: immunofluorescent ER aggregates occurred within the cytoplasm (Fig. 1b). This effect was also found in BY-2 cells treated with 75 μM NDGA (data not shown). As seen on electron micrographs (Fig. 2a,b), these aggregates corresponded to deformations of the ER network. In control maize root cells (Fig. 2a), ER appears like long tubular structures often paralleling the plasma membrane and running through

the cytoplasm. In NDGA-treated cells, convoluted ER membranes were often seen (Fig. 2b), along with 'fragmented' ER. This last impression is undoubtedly a consequence of planar sectioning through a cluster of ER.

Both in maize root cells and BY-2 cells (Fig. 1c), Golgi units stained by JIM84 were dispersed throughout the cytoplasm. In NDGA-treated cells, the same distribution was observed (Fig. 1d); no clustering of Golgi stacks was found whatever the NDGA concentration used. Electron microscopy did not reveal any significant changes of the basic arrangement of the Golgi stacks (Fig. 2c,d). Ultrathin sections of control cells (Fig. 2c) and 75 μM NDGA-treated cells (Fig. 2d), contrasted with the selective ZIO techniques, revealed a constant morphological feature of plant GA in both cell populations: the presence of stacks of membrane-bounded cisternae surrounded by a population of vesicles, with a polarity from the *cis* to the *trans* face.

BFA effects on maize root and BY-2 cell endomembranes performed under the same experimental conditions as those used for NDGA have been previously described and will be briefly recalled here: the Golgi units aggregate into several immunofluorescent compartments and undergo extensive vesiculation, leading to the complete deconstruction of the stacks. Subsequently, the ER network clumps into several aggregates [1,10,18]. Therefore, unlike mammalian cells, in plant cells NDGA does not disturb the organization of the Golgi apparatus, and does not exert effects analogous to BFA.

3.3. Effects of BFA and NDGA on lipid synthesis

Effects of BFA and NDGA on lipid synthesis in maize root cells were determined in a concentration range of 10–500 μM. Lipid labelling was decreased with both drugs, but inhibition of lipid synthesis was stronger with NDGA (Fig. 3). Lipid synthesis was almost totally stopped at 500 μM NDGA, coherent with the observation that cells died at this concentration.

For NDGA, polar and neutral lipid syntheses were similarly affected by the drug with a lower sensitivity of polar lipids at a low drug concentration (Fig. 4). However we found that sterol synthesis was significantly more sensitive to NDGA than any other lipid species. At a typical concentration of 100 μM NDGA, polar and neutral lipid syntheses were either not or only slightly decreased, whereas sterol synthesis was already inhibited by more than 50% (Fig. 4). Analyses by thin layer chromatography (TLC) revealed no accumulation of sterol precursors.

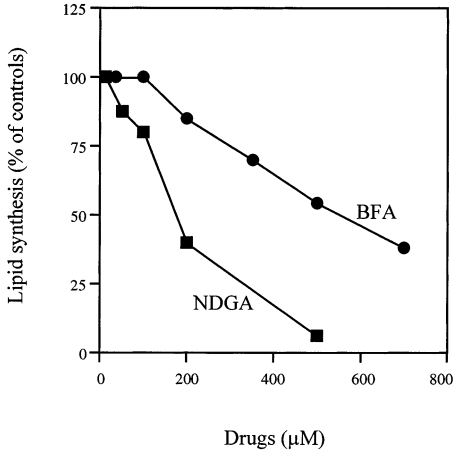

Fig. 3. Effect of BFA and NDGA on total lipid labelling of maize root apices. Acetate labelling was performed as reported in Section 2. Radioactivities of the total lipid extracts were determined by liquid scintillation counting.

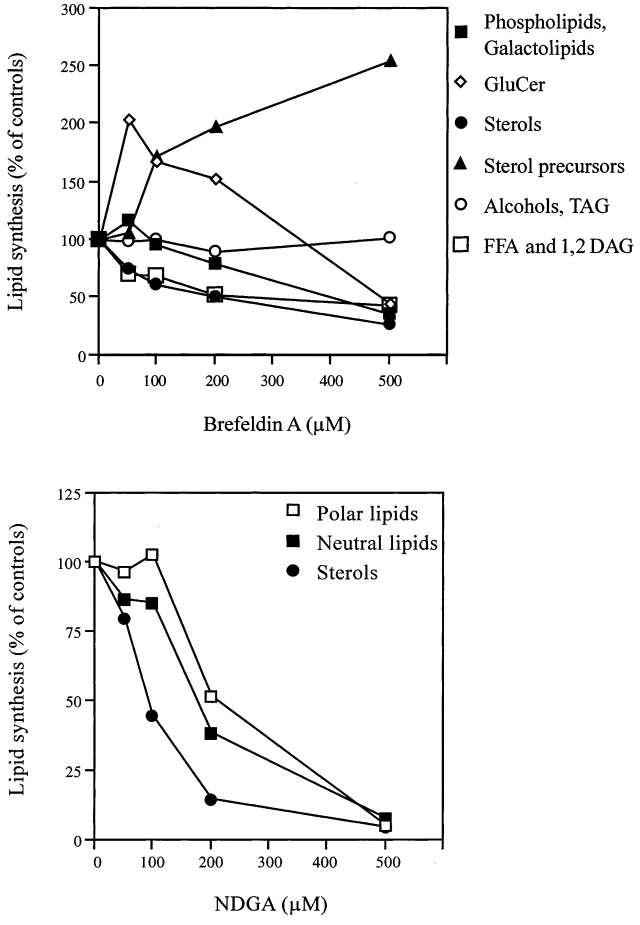

Fig. 4. Effect of BFA and NDGA on labelling of lipid species of maize root apices. Same experimental conditions as in Fig. 3. DAG: diacylglycerols; FFA: free fatty acids; GluCer: glucosylceramide; TAG: triacylglycerols.

Compared to NDGA, the effect of BFA on total lipid synthesis was milder, but sterol metabolism was found to be strongly disturbed. TLC analyses revealed that the ratio of sterol precursors to sterols shifted from about 0.4 to 1.8 for high BFA concentrations. A particular feature of BFA was that, at concentrations higher than 100 μM, we observed an accumulation of labelled sterol precursors (Fig. 4), suggesting that ultimate sterol metabolism was particularly sensitive to BFA treatment. To determine which step(s) is (are) especially sensitive to BFA and to look at kinetic parameters, a specific study has been undertaken on BY-2 cells. Phospholipid labelling was only affected when reaching 200 μM BFA with the strongest effects on phosphatidylserine synthesis. Glucosylceramide labelling was increased at 200 μM BFA, a situation already observed in animal cells [8], and strongly dropped at a higher drug concentration.

Our results emphasize a specific sensitivity of sterol metabolism to these lipophilic agents, and particularly to BFA.

4. Discussion

4.1. NDGA does not mimic BFA effects in plant cells

Using two plant materials, maize root apices and BY-2 cells, we found that NDGA affected the morphology of the ER but not that of the Golgi. In BFA-treated cells, the Golgi is altered and an ER aggregation is observed too, but only after the formation of Golgi aggregates, as a consequence of Golgi disturbance [1]. In NDGA-treated cells, ER modifications appeared as an early event and were not linked to Golgi apparatus dynamics. As the Golgi morphology is partly dependent upon transport from the ER, it may be surprising at first sight that the Golgi morphology is unaltered when the ER network is modified. To clarify this point, further studies of NDGA effects on ER function or NDGA effects on ER to Golgi membrane transport should be performed. After all, NDGA effects on ER structure may be completely independent of NDGA effects on transport machinery.

NDGA exerts a stronger inhibitory effect on total lipid synthesis than BFA. The morphological changes observed on the endomembrane system may be due to the fact that NDGA is a more general inhibitor of lipid synthesis than BFA, and that ER membranes are more dependent on total lipid synthesis than Golgi membranes. It suggests that the effects of BFA on plant Golgi involve additional molecular targets [4]. Conversely, it may be due to an inhibition specific to lipid species as discussed below.

4.2. BFA effect on lipid synthesis and structure–function relationship of Golgi membranes

Lipid analysis revealed a novel effect of BFA, i.e. the inhibition of phytosterol synthesis with accumulation of metabolic precursors. In animal cells, it has been found that cholesterol [24] is required for the formation of secretory vesicles from the *trans* Golgi network (TGN). In yeast, endocytosis is strongly dependent on sterol structure and particularly on the desaturation level of B rings [25]. Fatty acylation is also critical for fission of COPI-coated vesicles from the Golgi [26] and their subsequent fusion with target membranes [27]. Acylation of lysophosphatidic acid to phosphatidic acid (PA) was recently shown to be involved in fission of Golgi membranes [28], and in endocytosis and recycling of synaptic vesicles [29]. PA formation by phospholipase D was also found to be implicated in ER to Golgi transport [30] and in the dynamics of Golgi membranes [31,32]. In addition, phospholipase D was found to be activated by ARF and this, like the recruitment of COPI proteins, was BFA sensitive [33]. Finally, it seems that the homeostasis of PA and diacylglycerol is critical for Golgi secretory function [31,34]. Phospholipase A_2 has also been implicated in ER to Golgi traffic [35], intra-Golgi transport [36] and Golgi complex and TGN tubulation [37]. Interestingly, it has recently been shown that membrane susceptibility to phospholipase A_2 can be regulated by cholesterol [38].

Therefore, many links between the secretory pathway and lipid metabolism have been suggested in the literature. Several lipids and lipid-modifying activities seem to be key actors of membrane structure and dynamics required in membrane traffic and Golgi stack maintenance. In plant cells, it has been shown that specific lipid species including sterols follow the secretory pathway [7,39,40], and that the metabolic pathways of phosphatidylserine synthesis can be closely related to lipid sorting and trafficking [23,40]. In addition, we have recently observed that a specific inhibition of Δ^5 sterol synthesis (leading to an accumulation of sterol precursors such as cyclopropylsterols) can induce a fenestration of the Golgi apparatus in leek roots (Hartmann et al., submitted). It is noteworthy that BFA also led to an accumulation of sterol precursors. Direct or indirect, this inhibition is expected to have significant effects on membrane properties. Therefore, beside its potential effect on the COPI-dependent recycling pathway (as in animal cells), we can consider that the effect of BFA on Golgi dynamics in plant cells is also the consequence of a disturbance of lipid metabolism and membrane structure.

Acknowledgements: We thank Marie-Thérèse Crosnier for technical assistance on the immunofluorescence staining procedures and Dr. Spencer Brown for performing the cytometry analyses and critically reading of the manuscript.

References

[1] Satiat-Jeunemaitre, B., Cole, L., Bourett, T., Howard, R. and Hawes, C. (1996) J. Microsc. 181, 162–177.
[2] Ritzenthaler, C., Nebenführ, A., Movafeghi, A., Stussi-Garaud, C., Behnia, L., Pimpl, P., Staehelin, A. and Robinson, D. (2002) Plant Cell 14, 237–261.
[3] Saint-Jore, C., Evins, J., Batoko, H., Brandizzi, F., Moore, I. and Hawes, C. (2002) Plant J. 29, 661–678.
[4] Pimpl, P., Movafeghi, A., Coughlan, S., Denecke, J., Hillmer, S. and Robinson, D.G. (2000) Plant Cell 12, 2219–2235.
[5] Sanderfoot, A.A., Farhah, F.A. and Raikhel, N.V. (2000) Plant Physiol. 124, 1558–1569.
[6] Moreau, P. and Cassagne, C. (1994) Biochim. Biophys. Acta 1197, 257–290.
[7] Moreau, P., Bessoule, J.J., Mongrand, S., Testet, E., Vincent, P. and Cassagne, C. (1998) Prog. Lipid Res. 37, 371–391.
[8] Brüning, A., Karrenbauer, A., Schnabel, E. and Wieland, F.T. (1992) J. Biol. Chem. 267, 5052–5055.
[9] Slomiany, A., Grabska, M., Slomiany, B., Grzelinska, E., Morita, M. and Slomiany, B.L. (1993) Int. J. Biochem. 25, 891–901.
[10] Satiat-Jeunemaitre, B. and Hawes, C. (1992) J. Cell Sci. 103, 1153–1166.
[11] Orci, L., Tagaya, M., Amherdt, M., Perrelet, A., Donaldson, J., Lippincott-Schwartz, J., Klausner, R. and Rothman, J.E. (1991) Cell 64, 1183–1195.
[12] Peyroche, A., Antonny, B., Robineau, S., Acker, J., Cherfils, J. and Jackson, C.L. (1999) Mol. Cell 3, 275–285.
[13] Robineau, S., Chabre, M. and Antonny, B. (2000) Proc. Natl. Acad. Sci. USA 97, 9913–9918.
[14] Tagaya, M., Henomatsu, N., Yoshimori, T., Yamamoto, A., Tashiro, Y. and Mizushima, S. (1996) J. Biochem. 119, 863–869.
[15] Yamaguchi, T., Yamamoto, A., Furuno, A., Hatsuzawa, K., Tani, K., Himeno, M. and Tagaya, M. (1997) J. Biol. Chem. 272, 25260–25266.
[16] Drecktrah, D., de Figueiredo, P., Mason, R. and Brown, W. (1998) J. Cell Sci. 111, 951–965.
[17] Fujiwara, T., Takami, N., Misumi, Y. and Ikehara, Y. (1998) J. Biol. Chem. 273, 3068–3075.
[18] Couchy, I., Minic, Z., Laporte, J., Brown, S. and Satiat-Jeunemaitre, B. (1998) J. Exp. Bot. 327, 1647–1659.
[19] Satiat-Jeunemaitre, B. and Hawes, C. (2001) in: Plant Cell Biology, Practical Approaches (Hawes, C. and Satiat-Jeunemaitre, B., Eds.), pp. 207–233, Oxford University Press.
[20] Horsley, D., Coleman, J., Evans, D., Crooks, K., Peart, J., Satiat-Jeunemaitre, B. and Hawes, C. (1993) J. Exp. Bot. 44, 223–229.
[21] Napier, R.M., Fowke, L.C., Hawes, C., Lewis, M. and Pelhman, H. (1992) J. Cell Sci. 102, 261–271.
[22] Coba de la Peña, T. and Brown, S.C. (2001) in: Plant Cell Biology, Practical Approaches (Hawes, C. and Satiat-Jeunemaitre, B., Eds.), pp. 85–106, Oxford University Press.
[23] Vincent, P., Maneta-Peyret, L., Cassagne, C. and Moreau, P. (2001) FEBS Lett. 498, 32–36.

[24] Wang, Y., Thiele, C. and Huttner, W.B. (2000) Traffic 1, 952–962.
[25] Munn, A.L., Heese-Peck, A., Stevenson, B.J., Pichler, H. and Riezman, H. (1999) Mol. Biol. Cell 10, 3943–3957.
[26] Ostermann, J., Orci, L., Tani, K., Amherdt, M., Ravazolla, M., Elazar, Z. and Rothman, J.E. (1993) Cell 75, 1015–1025.
[27] Pfanner, N., Glick, B.S., Arden, S.R. and Rothman, J.E. (1990) J. Cell. Biol. 110, 955–961.
[28] Weigert, R., Silletta, M.G., Spano, S., Turacchio, G., Cericola, C., Colanzi, A., Senatore, S., Mancini, R., Polishchuk, E.V., Salmona, M., Facchiano, F., Burger, K.N.J., Mironov, A., Luini, A. and Corda, D. (1999) Nature 402, 429–433.
[29] Schmidt, A., Wolde, M., Thiele, C., Fest, W., Kratzin, H., Podtelejnikov, A.V., Witke, W., Huttner, W.B. and Söling, H.D. (1999) Nature 401, 133–141.
[30] Bi, K., Roth, M.G. and Ktistakis, T. (1997) Curr. Biol. 7, 301–307.
[31] Xie, Z., Fang, M., Rivas, M.P., Faulkner, A.J., Sternweis, P.C., Engebrecht, J. and Bankaitis, V.A. (1998) Proc. Natl. Acad. Sci. USA 95, 12346–12351.
[32] Siddhanta, A., Backer, J.M. and Shields, D. (2000) J. Biol. Chem. 275, 12023–12031.
[33] Ktistakis, N.T., Brown, H.A., Sternweis, P.C. and Roth, M.G. (1995) Proc. Natl. Acad. Sci. USA 92, 4952–4956.
[34] Henneberry, A.L., Lagace, T.A., Ridgway, N.D. and McMaster, C.R. (2001) Mol. Biol. Cell 12, 511–520.
[35] Slomiany, A., Grzelinska, E., Kasinathan, C., Yamaki, K.I., Palecz, D. and Slomiany, B.L. (1992) Int. J. Biochem. 24, 1397–1406.
[36] Tagaya, M., Henomatsu, N., Yoshimori, T., Yamamoto, A., Tashiro, Y. and Fukui, T. (1993) FEBS Lett. 324, 201–204.
[37] de Figueiredo, P., Drecktrah, D., Katzenellenbogen, J.A., Strang, M. and Brown, W.J. (1998) Proc. Natl. Acad. Sci. USA 95, 8642–8647.
[38] Klapisz, E., Masliah, J., Béréziat, G., Wolf, C. and Koumanov, K.S. (2000) J. Lipid Res. 41, 1680–1688.
[39] Moreau, P., Hartmann, M.A., Perret, A.M., Sturbois-Balcerzak, B. and Cassagne, C. (1998) Plant Physiol. 117, 931–937.
[40] Sturbois-Balcerzak, B., Vincent, P., Maneta-Peyret, L., Duvert, M., Satiat-Jeunemaitre, B., Cassagne, C. and Moreau, P. (1999) Plant Physiol. 120, 245–256.