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Changes of the secretory system of leaf epidermal and mesophyll cells during stress and programmed cell death

Turs Selga*

Laboratory of Plant Cell Biology, Faculty of Biology, University of Latvia, Kronvalda bulv. 4, Rīga, LV-1848, Latvia
*Corresponding author, E-mail: turs@lanet.lv

Abstract

Comparison was made between the effects of ethylene-induced programmed cell death and leaf senescence-caused programmed cell death on the Golgi apparatus and secretory pathway in tobacco leaves. Transgenic tobacco (*Nicotiana tabacum* L.) plants containing jellyfish green fluorescent protein (GFP) fused to rat sialyltransferase located in Golgi bodies and endoplasmic reticulum were used. Forty-day old plants were sprayed with Ethephon at concentrations of $5x10^{-3}$ M or $5x10^{-2}$ M. We observed cells from intact leaves and leaf sections cultivated in medium. Intact control plants and sections from expanding and mature leaves cultivated in Murashige and Skoog basal medium showed a high number of vectorially moving and oscillating Golgi bodies. Programmed cell death caused by leaf senescence or induced by Ethephon treatment caused a decrease of GFP fluorescence. Golgi bodies were located in the basal part of palisade parenchyma cells in senescent leaves but Ethephon treatment caused an increase of the number of Golgi bodies in the apical part of palisade parenchyma cells.

Key words: Golgi bodies, Ethephon, ethylene, Green Fluorescent Protein, *Nicotiana tabacum*, programmed cell death.

Introduction

Ethylene is a plant hormone involved in senescence and stress responses of plants. Wounding induces ethylene biosynthesis and ethylene is produced rapidly, with levels detectable within 30 min. Ethylene is an absolute requirement for the wounding response (Kende 1993; O'Donnell et al. 1996). The ethylene-releaser Ethephon causes an increase in the ethylene level in two ways: due to decomposition of Ethephon penetrating into leaf tissues and also by formation of ethylene from the endogenous pool of 1-aminocyclopropane-1-carboxylic acid by the action of ethylene-forming enzyme (Warner, Leopold 1969; Abeles 1973; Lieberman 1979; Romanovska et al. 1989; Ievinsh et al. 1990). We used Ethephon as a useful tool to increase the ethylene level in tissues and to stimulate programmed cell death in plant leaves.

The plasma membrane, vacuoles, Golgi complex and endoplasmic reticulum are involved in synthesis and binding of ethylene (Goodwin, Mercer 1983; Mayne, Kende 1986; Thompson et al. 1987; Bouzayen et al 1989; Crevecoeur et al., 1990; Hirayama ,

Alonso 2000). An increased level of ethylene causes changes in the number and activity of endoplasmic reticulum (ER) and Golgi apparatus (Osborne et al. 1985; Selga et al. 1985; Selga, Selga 2001).

Golgi apparatus and the secretory pathway have been recently visualised *in vivo* with the help of green fluorescent protein (GFP) targeted to different membrane proteins (Wee et al. 1998; Boevink et al. 1999; Hawes et al. 2001). However, the mechanisms of secretory system changes during programmed cell death are not clear.

The aim of the present work was comparison of the effect of ethylene-induced programmed cell death and leaf senescence-caused programmed cell death on the Golgi apparatus and secretory pathway in tobacco leaves.

Materials and methods

Transgenic tobacco (*Nicotiana tabacum* L.) plants contained jellyfish green fluorescent protein (GFP) fused to rat sialyltransferase located in Golgi bodies and endoplasmic reticulum (Andreeva 2000).

Plant seeds were disinfected with 70 % ethanol and plated on Murashige and Skoog (MS) basal salt medium (Sigma) containing 0.8 % agar Difco (Murashige, Skoog 1962). After incubation for 2 weeks at 20-22 ˚C, the seedlings were transferred to soil. Plants were grown in a greenhouse with a photoperiod of 17/7 h, 60 to 80 % relative humidity and 21±5 ˚C.

We compared expanding leaves, full grown leaves, yellowing leaves and leaves treated with Ethephon. We considered expanding leaves to be ones with an area not exceeding 1/4 of the area of full-grown leaves.

Treatment with Ethephon was realised in the following way: 40 day old plants were sprayed with a water solution of Ethephon (Cerone, Rhone-Poulenc Ltd.) at a concentration of $5x10^{-3}$ M or $5x10^{-2}$ M. Two and fourteen days after the treatment segments among veins from full grown leaves were cut and mounted on glass slides in a droplet of MS basal salt medium Sigma M5519 (4.4 g l^{-1}) and observed under a confocal microscope Zeiss CLSM 410. At the same time, we compared morphology of expanding leaves, full-grown leaves, leaves treated with Ethephon and senescent leaves.

The effect of wounding stress and cultivation in the MS basal medium was examined to evaluate the effect of cutting procedure on fluorescence intensity and number of Golgi bodies in cells of the upper epidermis and palisade mesophyll.

Transverse-sections (thickness = 40 to 50 μm) were cut with razor blade and mounted on glass slides in a droplet of MS basal medium M5519 (4.4 g l^{-1}). Sections were analysed by a confocal microscope at 30 min intervals during 5 h and after 48 h and compared with cells from intact leaves.

Confocal imaging with time-lapse scanning was performed on intact plant leaves emerged into the water on glass slides, leaf sections mounted into water or MS medium. Images were analysed with LSM Dummy (Zeiss) or Zeiss Image Browser 5.1 and cell size measured with Scion Image. We measured size of 300 epidermal cells from 10 leaf samples of control plants and 300 epidermal cells from 10 samples cultivated in MS medium for 48 h.

Location of Golgi bodies was analysed in 3 samples from 3 different leaves of each variant. Fluorescence intensity of samples was analysed with a Zeiss CLSM 410 using

the same objective and laser settings in the area of 512x512 pixels with software LSM Dummy (Zeiss).

Results

Comparing fluorescence, size and distribution of Golgi bodies in intact full grown leaves and leaf sections (d=20 mm), differences were not found. Intensity of fluorescence was variable among different leaves and leaf sectors in intact tissues. Cells showed intense fluorescence of ER in the cell cortex, and intense fluorescence of Golgi bodies. Some cells showed weak fluorescence of ER and Golgi bodies. In epidermal cells, most of the Golgi bodies were located among chloroplasts in the cortical region of the cell (Fig. 1A). The nuclear envelope also showed fluorescence and it was possible to observe nuclei in the lateral part of epidermal cells or at the bottom of the cell. Golgi bodies appeared to move between the nuclear envelope and the periphery of the cell.

In fully grown mesophyll cells of the spongy parenchyma, Golgi bodies were observed in the part of the cell close to epidermal cells (Fig. 1A). The view from above allowed to observe only 30 μm from the top of palisade parenchyma cells. This layer contained very few chloroplasts. In the deeper layer Golgi bodies were located among chloroplasts and close to the cell wall or tonoplast. Golgi bodies were randomly distributed in the mesophyll cells (Table 1). Golgi bodies were observed that appeared to move closely along chloroplasts. Movement was both vectorial and oscillatory.

When leaf segments were cultivated in MS medium, fast growth of leaf segments was observed. Area of leaf pieces during 48 h of incubation increased 2.6 times but the

Fig. 1 (in supplement). Cells of palisade parenchyma and upper epidermis of tobacco leaves in different stages of differentiation. Scale bar 20 μm. GFP fluorescence – green, chlorophyll fluorescence – red. A, Upper epidermis and palisade parenchyma cells of intact full-grown tobacco leaves. Intense green fluorescence of Golgi bodies and nuclear envelope (white arrow). B, Upper epidermis of intact mature tobacco leaves cultivated 24 h in Murashige and Skoog basal medium. High number of fluorescent Golgi bodies. Small Golgi body and large Golgi body (white arrows). C, Upper epidermis expanding tobacco leaves. High number of fluorescent Golgi bodies. D, Upper epidermis of leaves 2 days after the treatment of plants with Ethephon in a concentration of $5x10^{-3}$ M. Cells of upper epidermis with variable fluorescence and decreased number of Golgi bodies. E, Upper epidermis of leaves 2 days after the treatment of plants with Ethephon in a concentration of $5x10^{-2}$ M. Cells of upper epidermis with weak GFP fluorescence in the cortical cytoplasm (white arrow), no Golgi bodies are present. F, Upper epidermis of yellowing tobacco leaves. Cells of upper epidermis with weak GFP and chlorophyl fluorescence in the cortical cytoplasm, no Golgi bodies are present. G, Palisade parenchyma cells of expanding tobacco leaves. High number of randomly located fluorescent Golgi bodies (white arrow). H, Palisade parenchyma cells of intact mature tobacco leaves. Basal location of Golgi bodies increases. I, Palisade parenchyma cells of leaves 2 days after the treatment of plants with Ethephon in a concentration of $5x10^{-3}$ M. Apical location of Golgi bodies increases (white arrow). J, Palisade parenchyma cells of leaves 2 days after the treatment of plants with Ethephon in a concentration of $5x10^{-2}$ M. Weak GFP fluorescence in the cytoplasm, small number Golgi bodies are present (white arrow). K, Palisade parenchyma cells of yellowing tobacco leaves. Cells with weak GFP and chlorophyl fluorescence in the cytoplasm, basal location of Golgi bodies increases. L, Palisade parenchyma cells of leaves 2 days after the treatment of plants with Ethephon in a concentration of $5x10^{-3}$ M containing Golgi body in the vacuole (white arrow).

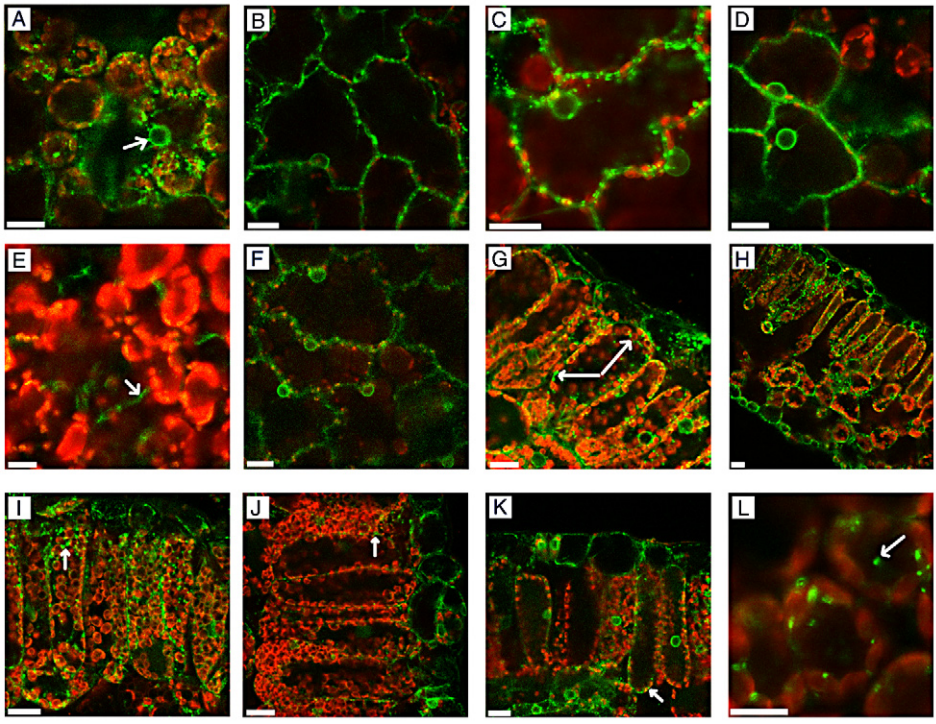
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Table 1. The percentage of the total number of Golgi bodies of a cell in the apical, medial and basal part of palisade parenchyma cells of tobacco leaves at different developmental stages. Values are the means ±SE of three different plants with three replicates each

Zone of the leaf	Expanding leaves (%)	Mature leaves (%)	Yellowing leaves (%)	Mature leaves treated with Ethephon ($5x10^{-3}$ M) (%)
Apical	29.3±4.3	24.6±5.2	17.3±2.8	46.2±3.7
Medial	40.1±5.2	42.7±6.3	36.2±4.5	38.1±2.9
Basal	30.6±3.1	42.7±4.1	46.5±3.9	16.7±3.1

average area of epidermal cells increased by 65 %. The fluorescence of these segments gradually increased during the first 3 h of cultivation. The increase was due to an increase of fluorescence of chloroplasts, Golgi bodies and cortical ER. Enhanced GFP fluorescence was stable for a week. Afterwards, it decreased till the level of control plants. This experiment showed that we can use transverse sections of leaves as models to analyse the structure of palisade parenchyma cells.

The number and size of Golgi bodies increased both in mesophyll cells and epidermal cells after 24 h of cultivation (Fig. 1B). Active fusing and splitting of Golgi bodies were typical. Many transvacuolar strands were formed in epidermal cells and small and bright Golgi bodies moved in different parts of both mesophyll and epidermal cells. Movement of Golgi bodies was similar to intact plants.

Expanding leaves were typical with a higher number and fluorescence of Golgi bodies in epidermis and palisade parenchyma than in full-grown leaves (Fig. 1C, G). An active joining and separation of Golgi bodies to nuclear envelope typically occurred. Golgi bodies were randomly distributed in the mesophyll cells (Table 1). Transvacuolar movement of Golgi bodies in epidermis was observed.

Fluorescence of yellowing leaves (Fig. 1F, K) varied. Different stages of loss of GFP fluorescence in cortical ER and Golgi bodies appeared. Fluorescence was observed in guard cells, at the bottom of epidermal cells and at the top of mesophyll (Fig. 1K). Epidermal cells contained a decreased number of Golgi bodies that showed oscillatory movement (Fig. 1F).

Large and disperse fluorescent bodies appeared in mesophyll cells. Sometimes they moved along chloroplasts and through transvacuolar strands. Transverse sections showed that the number of Golgi bodies is higher on the bottom of palisade mesophyll cells in comparison with the top of cells (Table 1). Different cells manifested different levels of decrease in secretory activity.

Treatment of plants with Ethephon in a concentration of $5x10^{-3}$ M did not alter the morphology and anatomic structure of mature leaves but caused gradual senescence of leaves for 3 weeks after treatment.

The response of epidermal cells was different. The first group of epidermal cells gradually lost fluorescence of cortical ER and the number of Golgi bodies decreased (Fig. 1D). In another group the number and size of Golgi bodies increased. Golgi bodies were typical in the apical part of mesophyll cells (Fig. 1D, Table 1). Most of them moved

between the vacuole and plasma membrane. Sometimes, Golgi bodies appeared in the vacuole (Fig. 1L).

The intensity of fluorescence of tissues gradually decreased during the first week but increased during the second week after treatment, due to a change of GFP fluorescence. At that time the number of mesophyll cells with Golgi bodies decreased. Large and loosely structured Golgi bodies moved between the vacuole and cell wall, close to chloroplasts and the inside of the vacuole. Co-localisation of Golgi bodies and chloroplasts became typical (Fig. 1I), appearing as a changing colour of Golgi bodies from green to yellow.

After treatment with a solution of Ethephon at a concentration of $5x10^{-2}$ M, death of leaf segments appeared but some cells survived. These remaining cells started active growth and division three days after the treatment. In this case, two days after treatment we observed weak fluorescence in the ER of epidermal cells, but fluorescence of tissues was weak. The result was loss of GFP in the mesophyll and weak fluorescence of chloroplasts for most of the cells (Fig. 1E, J). Seven days after the treatment fluorescence started to increase and several epidermal and mesophyll cells showed the presence of Golgi bodies in the periphery of cells. The leaf blade expanded actively during two following weeks and cells showed an increase of fluorescence that was similar to that in the control plants. We observed a large number of Golgi bodies in the cortical part of epidermal cells but a small number of Golgi bodies in the mesophyll cells. Golgi bodies moved along transvacuolar strands and in few cases appeared in the centre of the vacuole or moved from the vacuole to the cortical part of the cell.

At the same time, several groups of cells did not lose GFP fluorescence. Few epidermal cells showed an active transport of Golgi bodies along the cell wall and between the nucleus and the cell wall through transvacuolar strands and between the cortical part of the cell and vacuole. In mesophyll cells Golgi bodies were located between the tonoplast and chloroplasts. Sometimes they appeared near the cell wall. After 24 h and 48 h, cells showed the features described above.

Discussion

Location and movement of Golgi bodies in epidermis of control plants is similar to that previously described (Hawes et al. 2001). Observation of palisade parenchyma cells is difficult due to the fact that in thick samples (l >50 μm) a laser beam loses light intensity. Thus, in vivo it is only possible to observe thin leaves or upper and lower epidermis, spongy mesophyll and the top of palisade mesophyll cells.

Mesophyll cell cultures are widely used to study programmed cell death and terminal differentiation (Sheen 1995; Lam et al. 2001). This experimental system permits to observe changes in gene expression and proteins during stress-induced programmed cell death (Kovtun et al. 2000; Tena et al. 2001). The preparation of mesophyll cell cultures involves damage of the cell wall (Sheen 1995). The study of the secretory system of mesophyll cell culture during stress is restricted because Golgi bodies could be more involved in the formation of the cell wall.

Comparison of cells from leaf segments and segments cultivated in Murashige and Skoog basal medium with cells of intact plant leaves shows that wounding stress does not damage Golgi bodies and does not disturb the transport system of these cells. In all cases Golgi bodies are visible, and we observed their vectorial movement, indicating an

unchanged cytoskeleton.

In all observed samples we observed bright GFP fluorescence in the nuclear envelope. In addition, bi-directional moving of Golgi bodies from cortical cytoplasm to nuclei was typical, indicating that the surface of nucleus in tobacco leaves plays an important role in the secretory pathway. In *Toxoplasma gondii*, microtubule inhibitors or dithiothreitol have been observed to disrupt Golgi, causing swelling of the nuclear envelope (Hager et al. 1999).

A typical feature of programmed cell death of leaves either caused by terminal differentiation programme or induced by stress is the activation of proteolytic cascade (Lam et al. 2001; Tena et al. 2001). This can explain the decrease of GFP fluorescence intensity in all cases of observed programmed cell death.

However, 4 to 7 % of epidermal and palisade parenchyma cells retained GFP fluorescence intensity at a level similar to that in mature leaves. This shows that these cells retained high secretory activity and inhibited programmed cell death.

Golgi stacks can actively move along actin filaments and microtubules using motor proteins (Nebenführ, Staehelin 2001). In preparation for cell division, the Golgi stacks redistribute to the perinuclear cytoplasm, but during cytokinesis, this distribution changes and a higher density of Golgi stacks is found near the phragmoplast, the site of cell plate formation (Nebenführ et al. 2000).

Analysis of the distribution of Golgi bodies in cells of expanding and full-grown leaves as well as undergoing programmed cell death showed different locations of Golgi bodies (Table 1). Palisade parenchyma cells of expanding leaves showed random distribution of Golgi bodies. In contrast, yellowing and mature leaves with secretory activity at the basal part of the cell were typical. This suggests secretion of cell products to the apoplast and leaf veins. However, the data from expanding leaves are not precise due to the fast vectorial movement of Golgi bodies.

Ethephon in a concentration of $5x10^{-3}$ M induced activation in the secretory system in the apical part of the palisade parenchyma cells. The number of large Golgi bodies increased, suggesting that cells during stress excrete toxic metabolites into the apoplast and out of leaves. Co-localisation of chloroplasts and Golgi bodies became typical. This supports previous observations that ethylene induces the appearance of transport vesicles in the narrow (20 to 100 nm) space between chloroplasts and both cis and trans faces of Golgi bodies (Selga, Selga 2001).

Ethephon in a concentration of $5x10^{-2}$ M induced fast disappearance of GFP and chloroplast fluorescence. This can be explained by a hypersensitive response of cells.

The presented calculations of the number of Golgi bodies can show only trends of their distribution. Electron microscopic analysis of the location of Golgi bodies in mesophyll cells is required to diminish uncertainties due to vectorial movement of Golgi bodies and their impact on the number of visible Golgi bodies.

Programmed cell death induced with Ethephon differs from that of yellowing, in the direction of transport of secretory products. Yellowing causes transport towards leaf veins but Ethephon induces secretion towards leaf surface.

All observed cases of programmed cell death suggest that there exists a population of leaf cells that does not enter cell death programme.

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Lapu epidermas un mezofila šūnu sekretorās sistēmas izmaiņas programmētās šūnu bojāejas procesā

Tūrs Selga*

Augu šūnu bioloģijas laboratorija, Bioloģijas fakultāte, Latvijas Universitāte, Kronvalda bulv. 4, LV-1848, Rīga, Latvija
*Korespondējošais autors, E-pasts: turs@lanet.lv

Kopsavilkums

Darbā salīdzināja Goldži kompleksa un sekretorās sistēmas izmaiņas lapu novecošanas un etilēna izraisītās programmētās šūnu bojāejas procesā. Pētījumos izmantoti transgēni tabakas (Nicotiana tabacum L.) augi, kas satur zaļo fluorescento proteīnu (GFP), pievienotu pie žurku sialiltransferāzes, kas lokalizēta Goldži ķermenīšos un endoplazmatiskajā tīklā. Četrdesmit dienas vecus augus apsmidzināja ar etefonu $4x10^{-3}$ M vai $4x10^{-2}$ M koncentrācijā. Pētīja šūnas, kas atradās intaktu augu lapās vai lapu griezumos, kuri bija ievietoti Murišiges un Skūga sāļu barotnē. Visvairāk vektoriāli un oscilējoši kustīgu Goldži ķermenīšu bija intaktu augu, barotnē ievietotu augošu un izaugušu lapu griezumu šūnās. Programmēta šūnu bojāeja visos gadījumos izraisīja GFP fluorescences samazināšanos. Goldži ķermenīši visbiežāk bija novecojošu lapu zedeņu parenhīmas šūnu bazālajā daļā, bet etefona apstrāde izraisīja Goldži ķermenīšu skaita palielināšanos zedeņu parenhīmas šūnu apikālajā daļā.