Leaf morphology and anatomy of transgenic cucumber lines tolerant to downy mildew

M. SZWACKA[1]*, T. TYKARSKA[2], A. WISNIEWSKA[3], M. KURAS[2], H. BILSKI[4] and S. MALEPSZY[1]

Department of Plant Genetics, Breeding and Biotechnology[1] and Department of Plant Physiology[3], Warsaw University of Life Sciences, Nowoursynowska 159, PL-02776 Warsaw, Poland
Department of Plant Morphogenesis, University of Warsaw, Miecznikowa 1, PL-02096 Warsaw, Poland[2]
The Nencki Institute of Experimental Biology, PAS, Pasteura 3, PL-02093 Warsaw, Poland[4]

Abstract

The objective of the present paper was to investigate the reason of increased tolerance to the pathogenic fungus *Pseudoperonospora cubensis* found in transgenic cucumber (*Cucumis sativus* L.) lines 210 and 212 bearing 35S:cDNA *preprothaumatin II* gene construct. The tolerance investigation was accomplished by comparing the morphological and anatomical structure of plant leaves. The results obtained demonstrate that leaves of both lines exhibited some anatomical and morphological characteristics (*e.g.* wax load and composition, cuticle ultrastructure, ultrastructure of secondary wall, arrangement of mesophylll cells) which may be responsible for enhanced tolerance.

Additional key words: *Cucumis sativus* L., pathogenesis-related proteins, *Pseudoperonospora cubensis*, thaumatin II.

Introduction

Penetration of plant tissues is always a crucial step for plant-fungus interaction. Downy mildew, a major foliar disease in cucumbers (*Cucumis sativus* L.), is caused by the obligate biotrophic oomycete pathogen *Pseudoperonospora cubensis* (Berk. *et* Curt.) Rostovzev (Lindenthal *et al*. 2005). Before infecting host tissues, many parasitic fungi develop characteristic structures such as apressoria that are necessary for infection. One example is *P. cubensis*, which infects only the leaves of susceptible cucurbits. After infection, the pathogen colonizes the mesophyll, forming haustoria in paren-chymatic cells.

The epidermis and its cuticle are the plant's first line of defence against external influences. The outermost layer of the cuticle is composed of wax, whereas the insoluble polymers constituting the cuticle proper are deposited beneath (Sieber *et al*. 2000). Epicuticular wax and layered cuticle cover the cell wall of the epidermal cell. In addition to its role in reducing water loss through the epidermis, this outer layer has a major function in the interaction with plant pathogenic fungi (Sieber *et al*. 2000).

Moreover, plant combat fungal infections by the synthesis of a number of pathogenesis-related (PR) proteins. Previously, the expression of PR protein related genes in response to inoculation with the host pathogen was reported for grapevine, indica rice and *Brassica napus* (Kortekamp 2006, Nandakumar *et al*. 2007, Profotová *et al*. 2007).

Thaumatin II is a protein found in the West African fruit plant *Thaumatococcus daniellii* Benth that gives the plant its characteristic sweetness (Van der Wel and Bel 1980). Some efforts have already been made to express the *35S:cDNA preprothaumatin II* gene construct in potatoes (Witty 1990), tomatoes (Bartoszewski *et al*. 2003) and tobacco (Rajam *et al*. 2007). Transgenic cucumber lines with different levels of thaumatin II protein and sweet fruits have also been developed (Szwacka *et al*. 2002). Apart from the targeted change, these lines were also more tolerant to pathogenic fungus *P. cubensis*. A possible reason for this unexpected trait is the change of leaf structural barriers to the invading pathogen. Moreover, there is a high degree of homology between PR-5 family and thaumatin (Edreva 2005).

Tolerance of transgenic cucumber lines to downy mildew may be also due to activity of thaumatin-like PR protein, including members of the PR-5 family.

In the present study, leaf morphology and anatomy were analyzed in order to elucidate the nature of enhanced tolerance in two transgenic cucumber lines.

Materials and methods

Plants and growth conditions: The procedure used to transform cucumbers and the molecular characteristics of the resulting *35S:preprothaumatin II* cucumber lines have been described (Szwacka *et al.* 1996, 2002). In this study, we used T_3 and T_4 generations of two mildew-tolerant lines (210 and 212). The following terminology is used: a 'line' means the progeny of one independent transformation event after self pollination and a 'subline' represents the progeny of a chosen plant from the given line. Analysis was carried out on material derived from plants sown at the same time. The samples were prepared from plants at the same stage of development (23 nodes of the main shoot) that were free of infection. Plants were cultivated from spring to summer under standard conditions in a computer-controlled greenhouse. Irradiance was approximately 326 [W m^{-2} PAR], 16-h photoperiod, day/night temperature of 25/18 °C and air humidity approximately 80 %.

Leaf structure and ultrastructure: Leaf segments (1 × 2 mm) were excised from mature leaves (fourth or fifth node of the main shoot). The segments were excised from the leaf blade near the main veins between the second and third lateral veins, immediately fixed, embedded, sliced into semi-thin and ultra-thin sections and stained according to Mikula *et al.* (2005). Semi-thin

sections were photographed with a *Nikon* camera fitted on an *NU Zeiss* (Jena, Germany) light microscope. Ultra-thin sections were viewed with a *BS 500 Tesla* transmission microscope (Brno, Czech Republic).

Measurement of cell area and intercellular space: Measurements were carried out in triplicate, on at least three semi-thin leaf sections derived from a particular node (fourth or fifth) of three or four plants of investigated lines. First, the area occupied by whole parenchyma was measured (this area was considered to be 100 %). Second, the area occupied by cells was measured. The difference between these data represents the area of intercellular spaces. Semi-thin sections were measured under a light microscope (*LKB Carl Zeiss* Jena) fitted with a *PL-A642 Mega Pixel Fire Wire* camera. The data were analyzed with the help of the *LUCIA* computer program.

Statistical analysis: To evaluate differences in cell areas and intercellular spaces of leaf parenchyma of sublines and WT plants, data obtained from the *LUCIA* computer program were normalized by Bliss transformation (Bliss 1938). All calculations were carried out using the *Statgraphics Plus 4.1* for *Windows* software package.

Results

Leaf surfaces and cross-sections were microscopically examined in detail in order to determine whether there were any pre-existing structural barriers in the transgenic lines that might hamper fungal pathogen invasion.

Wax and trichomes were found on the epidermis in all of lines examined. The wax formed a film either with or without incrustation, or with some dust-like wax particles. In wild-type plants, there was a smooth, thin film on both sides of the leaf (Figs. 1*A,C,F*). In subline 210 04_5, there was also a thin wax film as in the wild-type plants, but on the abaxial side, there were minute, dense dust-like wax particles (Fig. 1*B*). In sublines 212 06_5 and 210 03_1, there was a wax cover on both sides of the leaf. In subline 212 06_5, the film was smooth and considerably thicker (Fig. 1*E*). In subline 210 03_1, the film was slightly thinner, and the epidermis was densely covered with wax particles (Fig. 1*D*). In subline 210 03_2, the wax film was densely encrusted with lobulo-granular deposits (Fig. 1*G*).

The ultrastructure of the cell walls was similar on both sides of the leaf. In the progeny of line 210, the

cuticle was electron-lucent, densely arranged in layers and had a small amount of wax particles on both surfaces (Figs. 2*D,E*). The cell wall was thickest in subline 210 04_1 (Fig. 2*D*). In the progeny of line 212, the cell walls were arranged in delicate layers and were about half as thick as in subline 210 04_1 (Figs. 2*G,H,I* compare with Fig. 2*D*). In subline 212 06_1, the cuticle was detached from the cell wall and covered with rather dense wax deposits (Fig. 2*H*). In subline 212 01_2, the cuticle was confined to areas covered by wax particles (Fig. 2*I*). In subline 212 06_3, the cuticle was continuous and gray, and was covered with numerous wax deposits. In wild-type plants, the cell walls were about one third as thick as in subline 210 04_1 (Fig. 2*F*). They were arranged in delicate, continuous layer on the surface of a thin electron-gray cuticle. The cuticle surface was covered with a few gray wax deposits.

Although the general mesophyll structure in all of the transgenic lines was similar and typical for cucumber plants, it differed in the packing density of mesophyll cells and intercellular space areas (Figs. 2*A,B,C*; Table 1).

Subline 210 04_3 had the loosest mesophyll structure because it had the smallest total cell area and the largest total intercellular space (Table 1). Cell area and
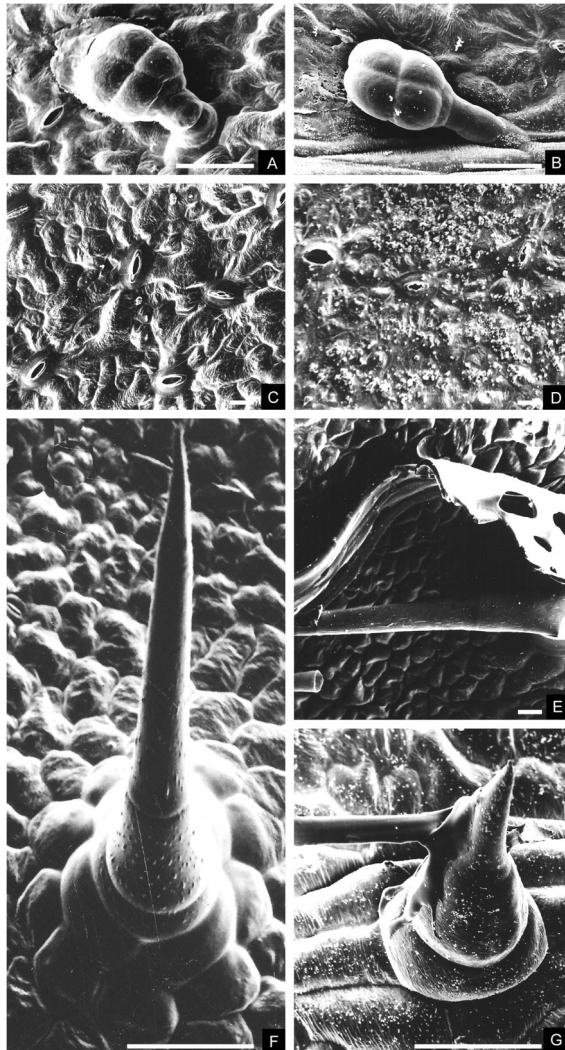

Fig. 1. Waxes on the leaf surfaces in the transgenic and WT plants. *A, C, F* - WT plants. *B, D, E, G* - transgenic plants. *A* - Adaxial leaf surface of WT plant: a fragment of smooth wax film at the trichome head and on the stomatal surface. *B* - Abaxial leaf surface of 210 04_5. Dense, coarse-grained wax structures. A trichome on a short stalk covered with smooth wax film. *C* - Adaxial leaf surface of WT plant. A thinner wax film without granular incrustation. *D* - Adaxial leaf surface of 210 03_1. A smooth, thinner wax film with granular incrustation underneath and on the epidermal cell surface. *E* - A patch of thick, smooth wax film on the adaxial leaf surface of 212 06_5. *F* - Ordinary trichome of WT plant with slightly, thinner wax film. *G* - Unusually dense, coarse-grained wax structures on the abaxial leaf surface of 210 03_2. *Bars*: *A, B* - 20 μm, *C* to *E* - 10 μm, *F, G* - 15 μm.

intracellular space were significantly higher in subline 210 04_3 than in wild-type plants. However, cell density was greatest in the progeny of line 212. Subline 212 06_2 had the densest palisade parenchyma and subline 212 01_1 had the densest spongy parenchyma (Table 1).

Table 1. Cell areas and intercellular space for palisade and spongy leaf parenchyma in transgenic and wild-type plants (Bliss transformed data; a,b,c,d - homologous groups, $P < 0.05$).

Lines	Palisade cell area	intercellulars	Spongy cell area	intercellulars
WT	1.06[b]	0.52[abc]	0.85[a]	0.72[b]
210 04_2	1.09[abc]	0.48[bcd]	0.84[ab]	0.73[ab]
210 04_3	0.92[d]	0.65[a]	0.62[b]	0.95[a]
210 04_4	1.03[abcd]	0.54[abcd]	0.76[ab]	0.81[ab]
212 01_1	1.13[ab]	0.44[cd]	0.92[a]	0.65[b]
212 06_2	1.20[a]	0.37[d]	0.88[ab]	0.69[ab]
212 06_3	0.96[cd]	0.61[ab]	0.77[ab]	0.80[ab]
212 06_5	1.09[abc]	0.48[bcd]	0.90[a]	0.68[b]
F	4.46	4.43	2.45	2.45

Discussion

In previous research, *35S:preprothaumatin II* cucumber lines were more tolerant to downy mildew than the wild-type plants *in vitro* (Szwacka *et al.* 2002). Moreover, our evaluation of these lines *in vivo* indicates that they are more tolerant to both forms of mildew (powdery and downy), and this phenotype is maintained in subsequent generative generations (not shown).

In the present study, we searched for pre-existing plant structural barriers by analyzing leaf morphology and anatomy in greater detail. We placed special emphasis on epicuticular wax load and leaf structure and ultrastructure. Contrary to a homogenous wax film found in the susceptible wild-type plants, the line 210 has shown high wax load as well as considerably high wax

incrustation. It implicates changes in wax composition. Changes of wax physical and chemical parameters might influence *P. cubensis* appressoria differentiation (Kolattukudy *et al.* 1995).

The cuticle is considered as a physical barrier to pathogens and also as a source of signals perceived by invading fungi (Chassot *et al.* 2007). The ultrastructure of the cuticle differed among the cucumber transgenic lines and in the wild-type plants. Research conducted by Chassot *et al.* (2007) shows that structural defects of the cuticle can be converted into an effective defense. Cuticle defects were especially observed in subline 212 06_2 and it was covered with only few wax deposits. Increased epicuticular wax load, however, was especially true for

subline 212 06_1, which exhibited changed cuticle structure. It has been shown in barley that components of the cutin act as signal molecules in both plants and pathogens. They also behave as biochemical cues to induce appressorium differentiation (Sieber *et al*. 2000). Therefore, lack of some of its components important for appressorium differentiation might be one of the reasons for its enhanced tolerance, especially in subline 212 06_2,
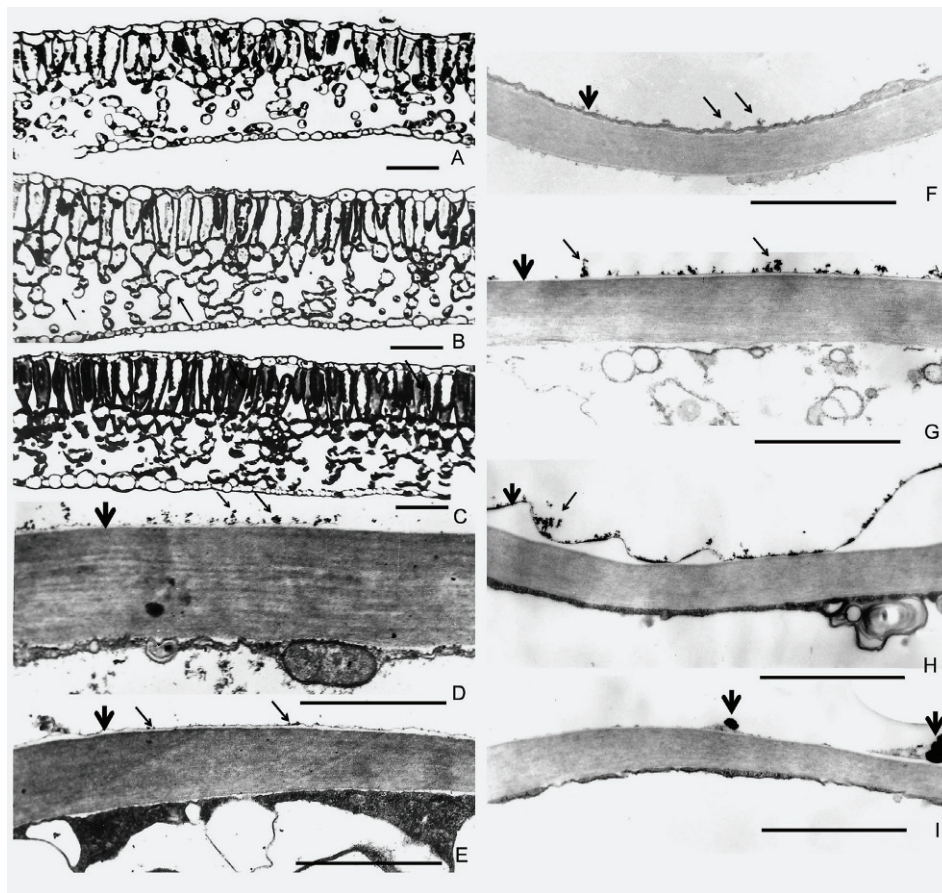

Fig. 2. Leaf anatomy (semi-thin transverse sections, *A* to *C*) and ultrastructure of the secondary cell wall of the epidermis (*D* to *I*). *A* - Loosely arranged mesophyll cells of WT plant. *B* - Large intercellular spaces (*thin arrows*) of 210 04_4. *C* - Closely packed mesophyll cells of 212 06_5, especially in the palisade parenchyma. *D* - Adaxial epidermis of 210 04_1, an unusually thick wall, visible dense layer arrangement. The surface of a thin, plicate, electron-lucent cuticle (*thick arrow*) shows visible granular electron-dense wax deposits (*thin arrows*). *E* - An electron-lucent cuticle (*thick arrow*), with fine, sparse wax deposits (*thin arrows*) of 210 04_4. *F* - The outer cell wall of the abaxial epidermis of WT plant with a visible cuticular layer (*thick arrow*) and fragments of homogenous, electron-gray wax (*thin arrows*). *G* - Adaxial epidermis of 212 06_3, a relatively thick wall with a visible cuticular layer (*thick arrow*) and electron flocculent-dense deposits (*thin arrows*). *H* - Adaxial epidermis of 212 06_1, the cuticle is detached from the cell wall (*thick arrow*). Its surface shows fairly dense, fine wax deposits (*thin arrow*). *I* - Adaxial epidermis of 212 01_2, the wall shows uneven thickness and a delicate layer arrangement. Cuticular layer fragments are clearly visible only at sites with electron-gray deposits (*thick arrows*). *Bars*: *A* to *C* - 25 μm, *D* to *I* - 1 μm.

which exhibited decreased amounts of cuticle.

The plant attempts to prevent infection by reinforcing of the cell-wall (Schmelzer 2002). In line 210, the secondary epidermal cell walls were up to three times thicker and were structurally more compact and ordered than in line 212 and the wild-type plants. In previous *in vitro* testing, the wild-type plants were susceptible to downy mildew (Szwacka *et al*. 2002a). Therefore, reinforced outer walls of line 210 might constitute other pre-existing host barrier, which can hamper downy mildew development.

Penetration of the plant tissue is a crucial step in plant-fungus interactions. *P. cubensis* is an obligatory parasite, whose zoospores encyst and germinate through leaf stomata (Lindenthal *et al*. 2005). Therefore, stomatal frequency and their accessibility to *P. cubensis* zoospores may determine the level of infection. However, statistical analysis revealed no significant differences in stomatal frequency between the mildew-tolerant transgenic lines and wild-type plants (not shown). Moreover, after infection, the pathogen colonizes leaf mesophyll, forming haustoria in parenchymatic cells (Cohen *et al*. 1989). In line 212, the intercellular spaces of the leaf mesophyll were smaller than in wild-type plants. Compact arrangement of the mesophyll cells in mildew-tolerant cucumber line could prevent the spread of infectious forms of the

attacking *P. cubensis* and therefore could hamper its invasion.

Plant combat fungal infections by the synthesis of a number of PR proteins. Tolerance of transgenic cucumber lines to downy mildew may be also due to activity of thaumatin-like PR proteins. There is a high degree of homology between PR-5 proteins and thaumatin (Edreva 2005), and the presence of recombinant thaumatin was confirmed in nearly all cell types of transgenic cucumber lines leaf (Szwacka *et al.* 2002b). Detailed biochemical investigation is, however, needed to resolve this question.

In conclusion, the increased tolerance of two analysed transgenic cucumber lines to downy mildew may result from unintended changes in morphological and anatomical leaf characteristics. The possible changes in transgenic plants as compared to the susceptible wild-type plants include: *1*) wax load and composition, *2*) cucticule ultrastructure, *3*) ultrastructure of the secondary cell wall of the epidermis and *4*) arrangement of the mesophyll cells. The combination of changes was different in each line. In each combination at least one change can enhance plant defence.

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