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Analysis of apple epidermis in respect to ontogenic resistance against *Venturia inaequalis*

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Abstract

In order to understand mechanisms of ontogenic resistance to apple scab, we analyzed various aspects of young and old leaves. We have introduced an apple plants cultivation system where *in vitro* propagated and rooting explants produce a genetically uniform population of apple (*Malus domestica* cv. Idared) plants. In this work, we demonstrate that apple plants produced in our cultivation system showed susceptibility to *Venturia inaequalis,* the cause of apple scab disease in young leaves and resistance in old leaves, which is similar to orchard situation. Our analysis shows that the cessation of epidermal cell expansion and shape formation coincided with the onset of ontogenic resistance in older leaves. Formation of specific cuticular lamellar structures did not coincide with ontogenic resistance onset. Further, chemical composition analysis of wax from young susceptible and old resistant leaves did not reveal specific compounds involved in ontogenic resistance. Differences in homogalacturonan content in cell walls in susceptible and resistant cells as well as decreased methylesterification of pectin in resistant leaves suggest that polysaccharide composition of the cell wall may play a role in mycelium growth and nutrition.

Additional key words: apple scab, cell wall, cuticle, homogalacturonan, *Malus domestica*, pectin.

Introduction

Plant epidermis is the main barrier that protects the plant body from the environment and pathogen attack. The cell wall of epidermal cells is the main mechanical barrier that protects plants from infection. Epidermal cells of aerial organs form a cuticle, which is a highly hydrophobic layer that is deposited on the surface of the cell wall; its primary role is protecting plant organs from water loss (Kerstiens 1996, Riederer and Schreiber 2001, Fernández *et al.* 2016). It is an important interface for plant-pathogen interactions (Serrano *et al.* 2014), which may be very specific and complex (Aragón *et al.* 2017).

Venturia inaequalis is a hemibiotrophic fungus that cause apple scab disease (González-Domínguez *et al.* 2017), and it is the most common fungal pathogen of apples. Upon infection, the fungus primarily colonizes the subcuticular space of the epidermis of young leaves and fruit (MacHardy 1996). It is believed that cell wall-degrading enzymes produced by the fungus degrade host cell walls in order to produce nutrients. Thus, cell wall components likely serve as the main carbon source for the fungus (Fothergill and Ashcroft 1955, MacHardy 1996). Cuticle degradation is essential for the infection process, and cutinase inhibition prevents infection (Köller 1991). A resistance based on the expression of various *R*-genes is the main tool in apple breeding (Jha *et al.* 2009). Ontogenic resistance which confers resistance to older leaves even in susceptible cultivars (MacHardy 1996) is a less studied factor. It is speculated that strengthened cuticle and cell

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Abbreviations: AIR - alcohol insoluble residue; ESEM - environmental scanning electron microscopy .
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wall in mature leaves and apoplastic pH play a role in ontogenic resistance (Jha *et al.* 2009). However, the exact mechanism of ontogenic resistance is not known.

In this work, we used environmental scanning electron microscopy (ESEM), transmission microscopy, and fluorescence microscopy to compare the adaxial surface of apple leaves from various ontogenic stages. We used *in vitro* propagated plants that were transferred to pots and cultivated under controlled conditions. Genetically homogeneous populations of susceptible apple plants mimicked the development of new leaves in orchards and their susceptibility to *V. inaequalis*. The aim of the paper was to describe characteristic changes of the cuticle structure and cell wall composition during leaf development and epidermal cell expansion in relation to the onset of ontogenic resistance to the infection by *V. inaequalis*.

Materials and methods

Plants and cultivation: Explants of *Malus domestica* Borkh. cv. Idared were cultivated on Murashige and Skoog medium (4.3 g dm^{-3} Murashige and Skoog salts, 100 mg dm^{-3} myo-inositol, 30 g dm^{-3} sucrose, 2 g dm^{-3} glycine, 0.38 µM thiamine, 2.96 µM pyridoxine, 4.06 µM nicotinic acid, 7 g dm^{-3} agar, pH 5.8) supplemented with 17.8 µM 6-benzylaminopurine in a growth chamber at a temperature of 21 °C, a 16-h photoperiod, and an irradiance of 120 µmol m^{-2} s^{-1}. To induce rooting in explants, four-week-old plantlets were transferred to Murasige and Skoog medium supplemented with 4.92 µM indole-3-butyric acid. After eight weeks, explants with extensive root systems were transferred to peat pellets and cultivated under non-sterile conditions in covered transparent beakers to increase air humidity. Transferred plants were acclimatized to ex *vitro* conditions for three weeks by gradually uncovering the transparent beakers. After four-weeks, plants were transferred to pots with soil, fertilized, and cultivated further under the environmental conditions mentioned above.

Growth rate of leaves was monitored for four weeks after transfer to pots. For growth analysis, leaves were numbered from the top of the stem to the base; the first leaf was labelled on day one after transfer and its position was recorded every two days. New leaves emerged every two days.

Plant infection: Conidia of *Venturia inaequalis* were washed from infected leaves of orchard plants. Conidia suspensions were stored at -20 °C for later use. For inoculation, plants in pots were sprayed with conidia suspension (a spore concentration of 2×10^5 cm^{-3}) and cultivated at 21 °C and a 98 % relative humidity in the dark for two days. Plants were then transferred to the above mentioned growth conditions. Visible infection developed within 7 days of cultivation. Evaluation was performed two weeks after infection. Infected leaves were sorted according to the following scale: class 0 = no attack, class 1 = 1 to 2 spots of infection per leaf, class 2 = 3 to 4 spots per leaf, class 3 = 5 to 7 spots per leaf, and class 4 = more than 7 spots per leaf. Disease severity for different leaf insertions was evaluated by the Townsend-Heuberger formula (Townsend and Heuberger 1943).

Tissue sectioning and cell wall staining: Leaf sections 150 - 200 µm thick were prepared using a hand microtome. Sections were immediately transferred to water and used for cell wall visualization. Cuticle was visualized by staining with 0.01% (m/v) *Auramine O* dissolved in 0.05 M Tris/HCl, pH 7.2. Leaf sections were stained for 1 h, rinsed with distilled water, and observed immediately under a confocal scanning microscope *LSM 880* (*Carl Zeiss s.r.o.*, Prague, Czech Republic; excitation at 405 nm, emission at 505 nm).

Leaf surface prints: A thin layer of nail polish was applied to the adaxial side of apple leaves, allowed to dry completely, and then removed using forceps. The leaf surface print was mounted in water and observed with an *Provis AX70* microscope equipped with a *Nomarski DIC* unit (*Olympus*, Tokyo, Japan).

Environmental scanning electron microscopy: Selected parts of leaves were observed using the low temperature method for ESEM developed for electron beam observation of unfixed wet samples without metal coating (Neděla *et al.* 2012, 2016a). Leaves were cut in 4 - 8 mm^2 segments and placed in 2 mm^3 of water on a flat cylindrical brass sample holder of the Peltier stage. The low temperature method for ESEM protocol according to Neděla *et al.* (2015) was used. The observation conditions were: air pressure 150 Pa and sample temperature -18 °C. All experiments were performed on *FEI ESEM QUANTA 650FEG* (*FEI*, Hilsboro, OR, USA); a beam accelerating voltage of 10 kV, a probe current of 26 pA; the distance between the sample surface and the bottom surface of the gaseous secondary electron detector was 8 mm).

Cuticle chemical analysis: Samples classified as young were comprised of the three youngest unfolded leaves. Samples classified as old were comprised of the fourth to sixth leaves. Leaf cuticles were removed by immersing each leaf in a Petri dish with 10 cm^3 of chloroform (10 s for adaxial and 10 s for abaxial side of the leaf). Chloroform was evaporated to dryness and the amount of solvent (hexane) with the added inner standard (10 ng mm^{-3} 1-bromoeicosane) was normalized to the leaf surface area, which was measured by placing leaves on a paper, tracing their outlines, cuting out leaf shapes, and weighing.

Chemical analyses were performed using a two-dimensional gas chromatograph with time-of-flight mass spectrometric detection (*GC×GC-TOFMS*; *Pegasus 3D*, *Leco*, St. Joseph, MI, USA), equipped with a combination of non-polar *ZB-5MS* (30 m, id 0.25 mm, 0.25 µm film thickness; *Phenomenex*, Torrance, CA, USA) and medium polarity *RTX-50* (1.5 m, id 0.1 mm, 0.1 µm film thickness; Restek, Bellefonte, PA, USA) columns. A split/splitless port was heated to 250 °C. The temperature program for the primary column was 50 °C (1 min) to 320 °C (20 min)

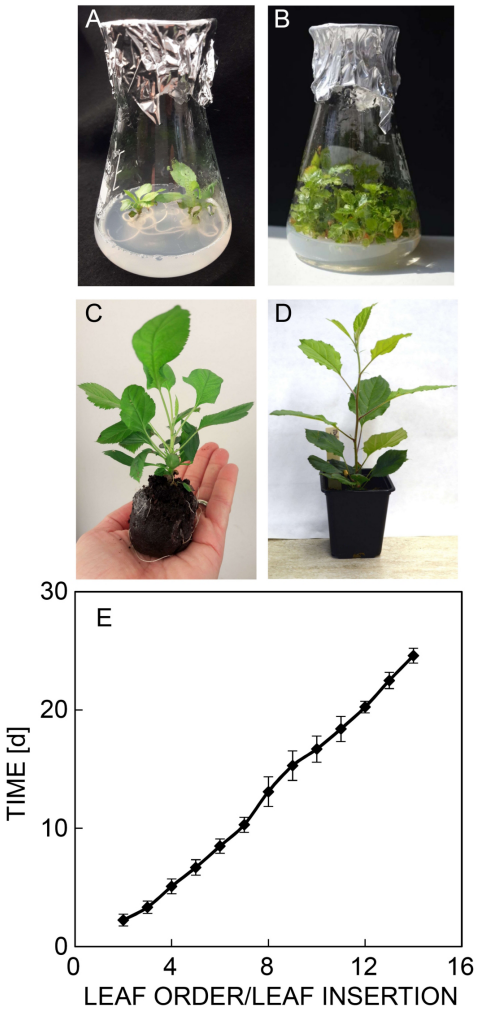

Fig. 1. *Malus domestica* cv. Idared cultivation system. *A* - Plants cultivated *in vitro* from explants. *B* - Plantlets transferred to a root-induction medium to induce root development. *C* - Plantlets transferred to peat pellets to produce an extensive root system. *D* - Plantlets cultivated in pots with soil. *E* - Gradul development of leaves in plants cultivated in pots. Means ± SEs, $n = 10$.

at 8 °C min^{-1}, the secondary column was set 10 °C higher. Chemical identifications were based on comparisons of mass spectrometry fragmentation patterns and retention characteristics (Kovats indices) of detected analytes with those reported in literature.

Cell wall pectin analysis: Analysis was performed of insertion one, two (young), and seven (old) leaves using an alcohol-insoluble residue (AIR) prepared as follows: 100 mg (f.m.) of ground samples was submerged into 96 % (v/v) ethanol and incubated at 70 °C for 30 min. The pellet was then washed with 100 % ethanol, twice with chloroform (100 %):methanol (100%) (2:1, v/v), followed by successive washes with 65, 80, and 100 % ethanol. The remaining pellet of alcohol insoluble residues (AIR) was dried in a fume hood overnight at room temperature.

Dry mass of each sample was measured. After saponification of the AIR (5 mg) with 0.05 M NaOH, the supernatant containing methyl ester released from the cell wall was separated by centrifuging (15 000 *g*, 15 min, room temperature) from the pellet with polysaccharides. Pectins were extracted from the pellet with 1 % (m/v) ammonium oxalate at 80 °C for 2 h as described by Krupková *et al.* (2007), Mouille *et al.* (2007), and Neumetzler *et al.* (2012). Uronic acid was then quantified by colorimetry using the meta-hydroxydiphenyl-sulfuric acid method as described by Blumenkrantz and Asboe-Hansen (1973). Methyl ester was quantified from the NaOH supernatant with a colorimetric assay using an enzymatic oxidation of methanol (Klavons and Bennett 1986).

Results

We established an apple plant cultivation system, where *in vitro* propagated apple explants produced young apple plantlets of uniform genotype and age irrespective of the season. *In vitro* explants (Fig. 1*A*) were first induced to form roots *in vitro* (Fig. 1*B*). Plantlets were transferred to peat pellets (Fig. 1*C*) and then to pots with soil (Fig. 1*D*) and cultivated under the same environmental conditions

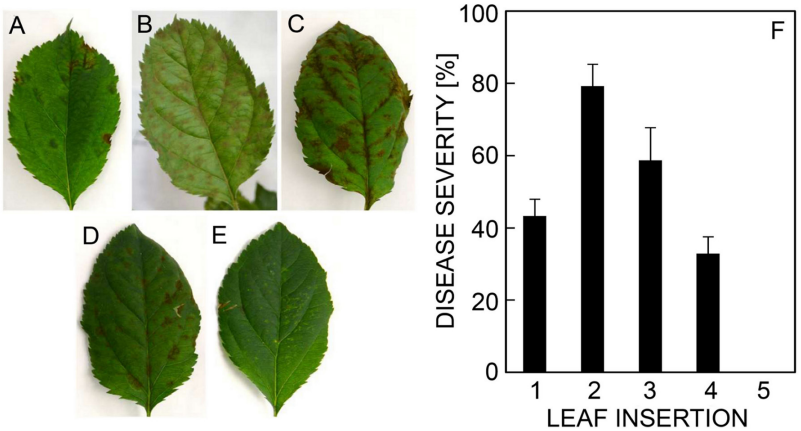

Fig. 2. *Venturia inaequalis* disease severity in leaves after two weeks of infection. *A* - Leaf insertion 1 (the first unfolded leaf), *B* - leaf insertion 2, *C* - leaf insertion 3, *D* - leaf insertion 4, *E* - leaf insertion 5. Leaf insertion refers to the moment of the first day of the infection. *F* - Disease severity quantification. Means ± SEs, $n = 31$.

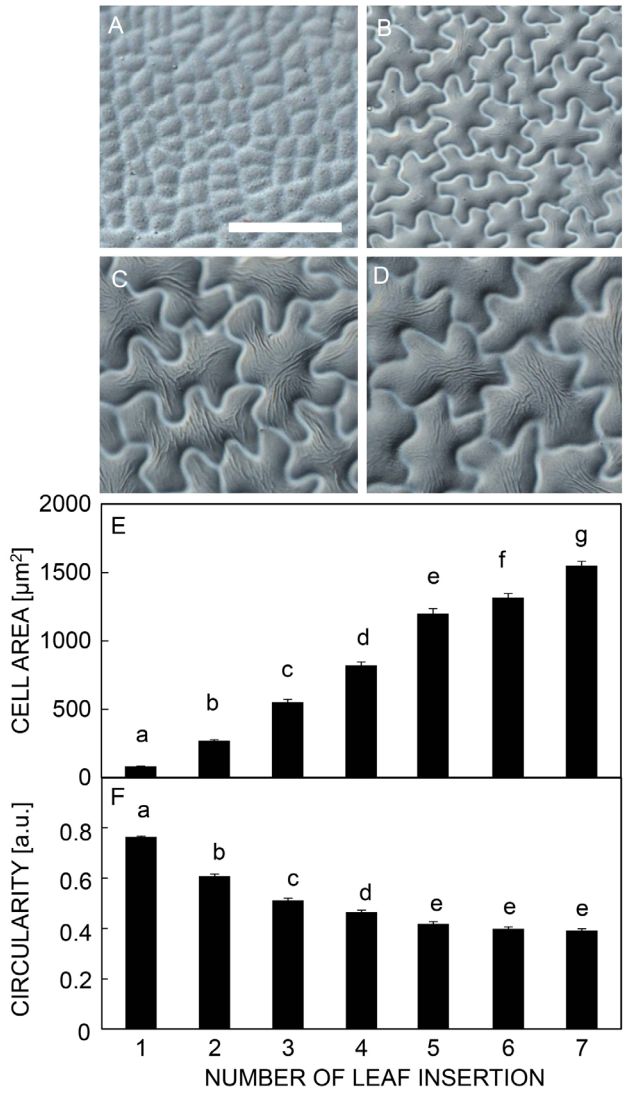

Fig. 3. Adaxial epidermal pavement cells of leaf insertion 1 (the first unfolded leaf) (*A*), leaf insertion 3 (*B*), leaf insertion 5 (C), and leaf insertion 7 (*D*). Nail polish imprints observed under *Nomarski DIC*; *scale bar* 50 µm. Cell area quantification (*E*). Circularity quantification (*F*). Means ± SEs, $n = 10$. Different letters indicate significant differences according to *ANOVA*.

during the whole process. Plantlets underwent a rapid growth and leaf development; new leaves developed every two to three days of cultivation (Fig. 1*E*). We used this cultivation system to study surface characteristics of leaves in respect to *V. inaequalis* infection.

Apple plants were infected with *V. inaequalis* in controlled conditions. *Venturia inaequalis* susceptibility and the onset of ontogenic resistance of leaves was comparable to leaf development in spring in orchards. As shown in Fig. 2*B*, the leaves most susceptible to *V. inaequalis* infection were the first to fourth youngest unfurled leaves. The fifth leaf showed an almost complete ontogenic resistance. In susceptible young leaves, a large mycelium was formed during subsequent cultivation (Fig. 2*A*).

In order to understand the ontogenic resistance onset, we compared growth parameters of young leaves susceptible to the infection to older leaves showing ontogenic resistance. Nail polish surface prints showed that the epidermal cells of the first unfolded leaf (leaf insertion one) had isodiametric shape (Fig. 3*A*). During expansion, typical jigsaw puzzle-like shapes were apparent in the second leaf, and more expanded lobes were formed in the third leaf (Fig. 3*B*). Cells further expanded to reach their final shape in the fifth and older leaves (Fig. 3*C*,*D*). Measurements showed that epidermal cells underwent a rapid expansion in young leaves (Fig. 3*E*). The expansion slowed down in the fifth leaf, but the cell surface area increased during the next development (Fig. 3*E*). A cell shape, expressed as a circularity of epidermal cells, shows that the rapid expansion was associated with a rapid formation of puzzle-like shapes of epidermis (Fig. 3*F*). In slowly expanding cells in the fifth and older leaves, the circularity parameter change was statistically insignificant, which suggests that the shaping process was finished (Fig. 3*F*).

We analyzed cell surface characteristics of adaxial epidermal cells. Nail polish surface prints revealed that isodiametric epidermal cells in the first leaves had an unstructured cuticle deposited on their surface (Fig. 4*A*). However, after the onset of a rapid expansion and formation of the first cell lobes in the second leaves (Fig. 4*B*), the cuticle formed parallel multiple lamellae, which were already abundant in the third leaf (Fig. 4*C*). As cells expanded, cuticle lamellae became more pronounced (Fig. 4*D*,*E*). The seventh leaf was the oldest analyzed (Fig. 4*F*), whose epidermal cells were covered with a fully developed lamellar cuticle. Fig. 4*L* shows quantification of the presence of cuticle lamellae confirming its absence on the first leaves.

To confirm our observations and to increase the resolution, we used ESEM for adaxial epidermis observation. The ESEM analysis confirmed the presence of an unstructured cuticle in the first leaves (Fig. 4*G*) and the presence of a lamellar cuticle in expanding epidermal cells (Fig. 4*H*,*I*).

To confirm that lamellar structures observed using nail polish surface prints and ESEM are cuticle structures, we detected the cuticle in cross-sections of leaves with lamellae. *Auramine O* staining confirmed that the observed lamellar structure is the cuticle. In young leaves, the cuticle formed a continuous smooth layer (Fig. 4*J*) whereas typical lamellae were formed in older leaves (Fig. 4*K*).

We analyzed the chemical composition of surface organic solvent-extractable waxes of cuticle from young susceptible and old resistant leaves in order to identify qualitative changes involved in ontogenic resistance. Chemical analysis allowed us to identify a list of compounds forming wax and cuticle, however, none of the identified compounds significantly accumulated in old leaves (Fig. 5 Suppl.). Significantly higher amounts of tetracosan-1-ol, heptacosan, and α-tocopherol were detected in fractions from young leaves (Fig. 5*B*).

To understand qualitative changes in cell wall

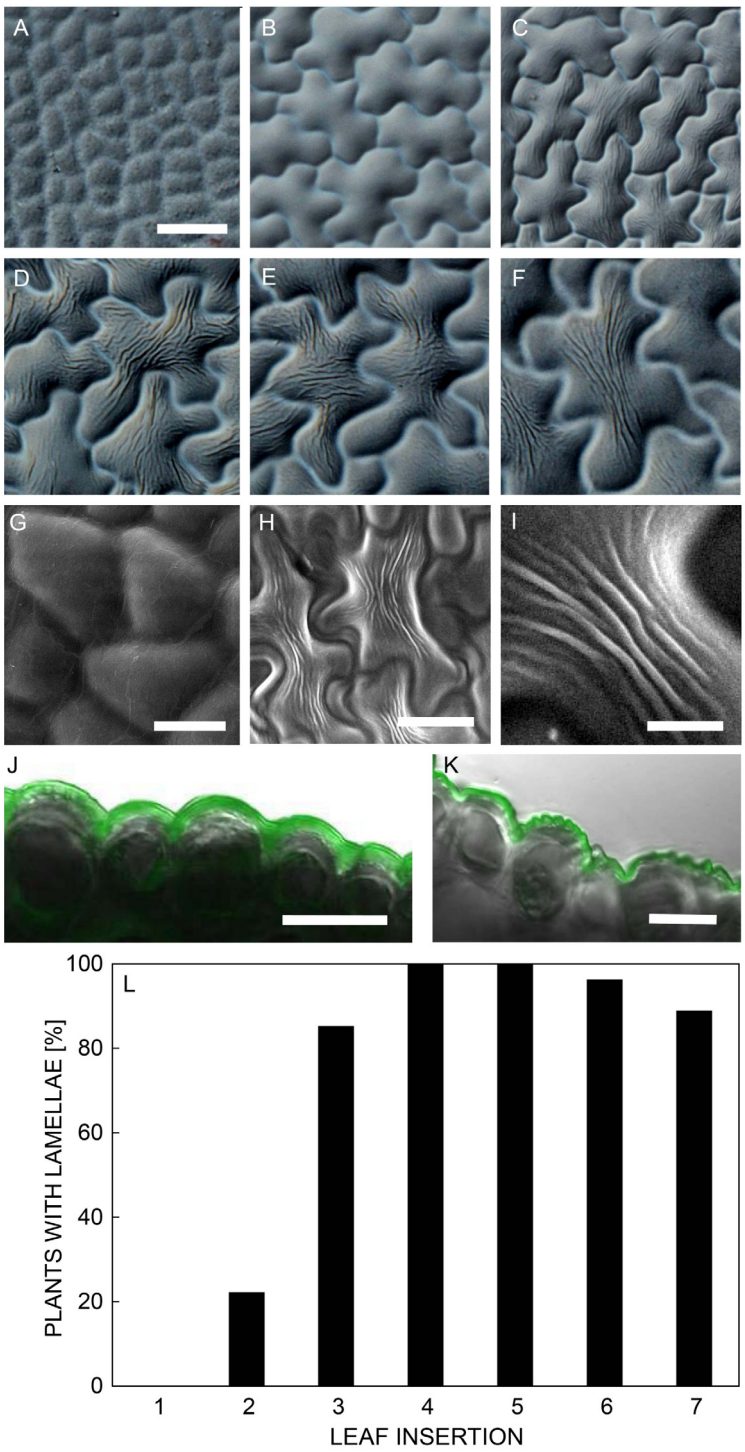

Fig. 4. Cuticle structure of adaxial epidermal cells observed in nail polish imprints and *Nomarski DIC*: *A* - leaf insertion 1 (the first unfolded leaf), *B* - leaf insertion 2, *C* - leaf insertion 3, *D* - leaf insertion 4, *E* - leaf insertion 5, *F* - leaf insertion 7. Cuticle structure of adaxial epidermal cells observed using environmental scanning electron microscopy. *G* - leaf insertion 1, *H*,*I* - leaf insertion 5. Transversal sections of adaxial epidermis made with a hand microtome and stained with *Auramine O* (green fluorescence). *J* - leaf insertion 1, *K* - leaf insertion 5. *Scale bars*: 25 µm (*A*-*F*), 10 µm (*G*, *J*, *K*), 20 µm (*H*), 5 µm (*I*). *L* - Quantification of cuticle lamellae incidence in leaves of various insertions (n = 27).

composition involved in ontogenic resistance, we analyzed the pectin content of cell walls in young susceptible and old resistant leaves. The amount of galacturonic acid extracted from mature leaves was significantly higher in old/mature leaves (Fig. 6*A*) Despite this, the amount of extractable methanol was not significantly different in young and old

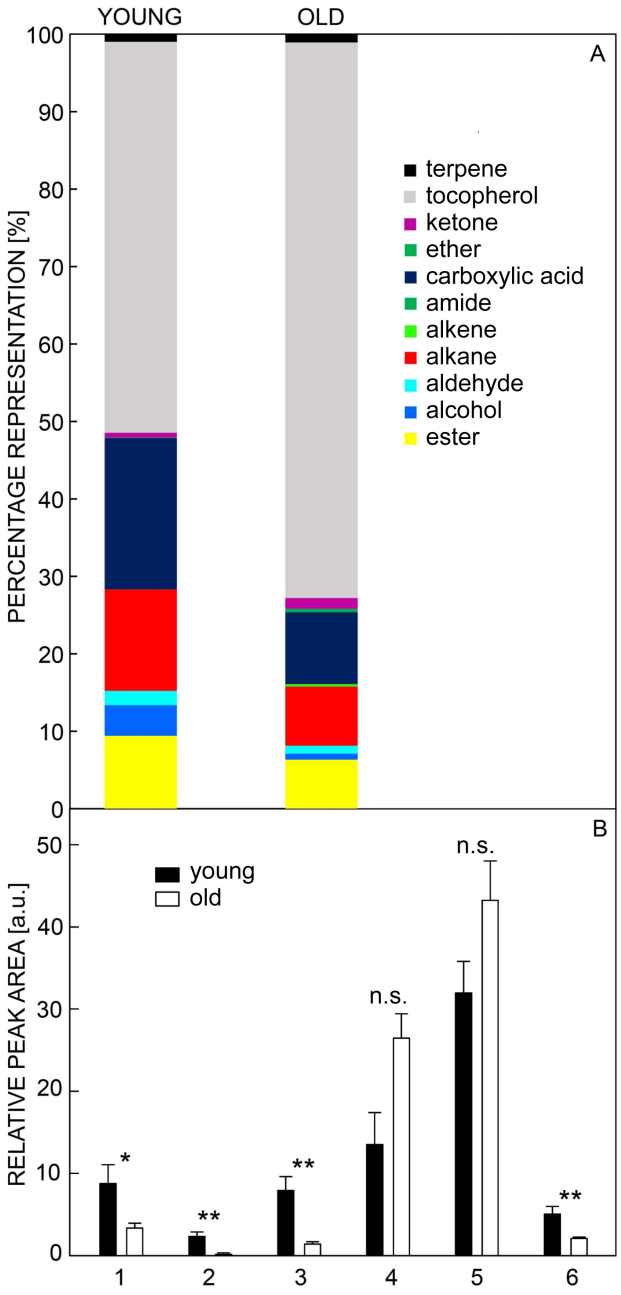

Fig. 5. Chemical analysis of apple leaf cuticle. *A* - Percentage of various compounds in dry mass of cuticle of young (leaf insertion 1 - 3) and old (leaf insertion 4 - 6) leaves. *B* - Content of selected compounds in cuticle. Comparison of young and old leaves: 1 - hexadecanoic acid, 2 - tetracosan-1-ol, 3 - heptacosane, 4 - δ-tocopherol; 5 - γ-tocopherol; 6 - α-tocopherol. Relative peak area - (peak area of analyte) to (peak area of internal standard). Means ± SEs, $n = 8$, significant differences at * - $P < 0.05$ and ** - $P < 0.01$, respectively, N.S. - non-significant, (*ANOVA*).

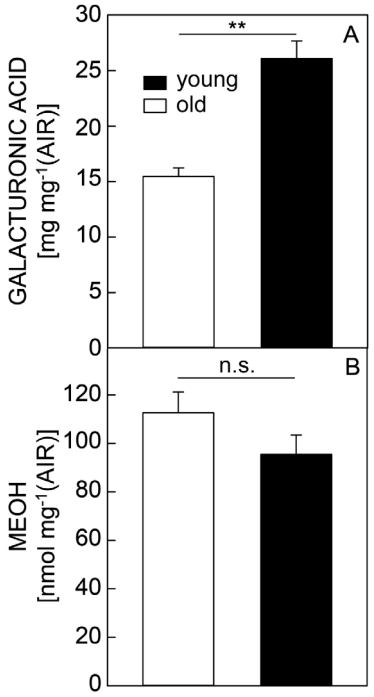

Fig. 6. Analysis of pectin content of young (leaf insertions 1 and 2) and old (leaf insertion 7) leaves. *A* - Galacturonic acid detected in the ammonium oxalate fraction of the cell wall. *B* - Methanol content in the cell wall extract. AIR - alcohol insoluble residue, MEOH - methanol. Means ± SEs, $n = 11$. ** - significant differences at $P < 0.01$, N.S. - non-significant, (Student's *t*-test).

leaves (Fig. 6*B*). This result suggests that pectin content increased in mature leaves. Importantly, the insignificant difference in extractable methanol amount suggests that pectin in old/mature leaves was extensively cross-linked through free carboxylic acid residues that were not blocked by methyl residues.

Discussion

Apple scab disease caused by the ascomycete *Venturia inaequalis* is the most common apple disease (MacHardy 1996). Various cultivars differ in *V. inaequalis* resistance. Several genes of resistance are known to confer apple resistance to *V. inaequalis* (Jha *et al.* 2009). Older leaves develop ontogenic resistance that confers resistance to older leaves (MacHardy 1996), which is linked to specific gene expressions (Gusberti *et al.* 2013). We have shown that young leaves of quickly growing apple plants of cv. Idared in our cultivation system were highly susceptible to *V. inaequalis* infection whereas older leaves were less susceptible, with the fifth leaf developing ontogenic resistance. This cultivation system has also other benefits such as season-independent propagation, genetically uniform plant material, and controlled cultivation conditions.

Growth characteristics analysis of adaxial pavement cells involved cell area and cell circularity measurements. Cell expansion (increasing the cell area) coincided with the formation of characteristic lobes of epidermal cells

(a decreasing circularity). The decline of cell expansion was first detected in the fifth leaf. The shape formation process (circularity) was finished in the fifth leaves. Therefore, the cell shape formation and the cell expansion are parameters, which coincided with the onset of ontogenic resistance to *V. inaequalis*.

We used our cultivation system to search for factors associated with ontogenic resistance. Here, we show that cuticle forms specific structures in growing and fully developed leaves. These structures (parallel cuticle folds forming characteristic lamellae) were formed shortly after the onset of epidermal cell expansions. Cuticle lamellae were present in all analyzed leaves except the youngest unfolded leaf. The presence of cuticular structures were confirmed also by the analysis of epidermis surfaces by ESEM. This method allows the observation of native, unfixed plant material, which excludes a possibility that cuticular structures observed using surface nail polish prints were result of plant surface damage (Neděla *et al.* 2016b). Cuticular lamellae were observed also by Bringe *et al.* (2006), who noted their presence in the youngest apple leaves, and reported their gradual disappearance in older leaves. The contrasting observation of the incidence of cuticle surface structures may be explained by different plant material used - apple seedlings of Golden Delicious (Bringe *et al.* 2006) and rooted explants of Idared cultivars (our study).

Hydrophobic molecules of cutin and waxes, as well as epicuticular wax crystals confer plant surfaces with a hydrophobic barrier and specific structural properties (Yeats and Rose 2013). Intact cuticle plays an important role in plant defense (Aragón *et al.* 2017). Although cuticle functions as a barrier for pathogens entry, fungal pathogens often breach the cuticle using specialized mechanisms. Inhibition of cutinase prevented *V. inaequalis* infection suggesting enzymatic hydrolysis of cuticle as an important factor for successful infection (Köller 1991).

The chemical analysis of cuticle from young susceptible and old resistant leaves allowed us to identify a number of compounds of the hydrophobic cuticular structure. These waxes consist of a complex mixture of free fatty acids, alcohols, aldehydes, ketones, esters, squalene, and tocopherols. Significantly higher amounts of palmitic acid, tetracosan-1-ol, and heptacosan were detected in young cuticles. Moreover, tocopherols were present in cuticles of both young leaves and old leaves; α-tocopherol showed a significantly increased content in young leaves. In leaf cuticular waxes, we detected three different isomers of tocopherols (α-, γ- and δ-, see Fig. 1 Suppl.). In young leaves, the total tocopherol content corresponded to 50.47 % of all wax compounds whereas in older leaves, a relative amount of the total tocopherol content increased up to 71.71 %. γ-Tocopherol was most abundant of all the tocopherols with 63 % in young and 60.2 % in old leaves. Only α-tocopherol was previously reported in apple leaves (Bringe *et al.* 2006). This is the first description of δ- and γ-tocopherols in leaf waxes of apples. In plant leaves, mostly α-tocopherol was reported whereas γ- and δ-tocopherols were described in plant seeds (Szymańska and Kruk 2008). Within *Rosaceae* family, epicuticular waxes of *Rubus ideaus* contain a similar composition of tocopherols, with γ-tocopherol being the most abundant isomer (Shepherd *et al.* 1999). We were not able to identify any unique compound present specifically in old leaves.

Our analysis of homogalacturonan content revealed that old leaves contained a higher homogalacturonan content than young leaves. Further, because the amount of released methanol was comparable in young and old leaves, we can assume that pectin in older leaves was less methylesterified. The importance of pectin modification in cell walls is currently being studied. Pectin molecules are transported in a highly methylesterified state into the cell wall. Here, pectin can be demethylesterified by cell wall enzymes pectin methylesterases. Classical models suggest that this leads to crosslinking pectin molecules through Ca^{2+} bonds, which increases the stiffness of the cell wall as shown experimentally by Hongo *et al.* (2012). On the other hand, measurements of cell wall mechanical properties using atomic force microscopy revealed that cell walls containing pectin with a lower degree of methylesterification are softer (Peaucelle *et al.* 2011, 2015). It seems that the remodeling of pectin in the cell wall is more complex than expected in classical models, and the influence of pectin methylesterification may be cell type specific. For example, under specific conditions, demethylesterified pectin may be more susceptible to enzyme degradation. It is now clear that pectin polysaccharides and their degree of methylesterification are associated with cell wall extensibility during plant cell growth, albeit the exact mechanism is not known (Wolf and Greiner 2012, Levesque-Tremblay *et al.* 2015, Hocq *et al.* 2017). The decreased methylesterification of pectin in old apple leaves coincided with the decline of epidermal cell shape formation and expansion, and increased resistance to *V. inaequalis*. Our observation thus suggests a cell wall reinforcing role of pectin demethylesterification.

Cell wall fragments produced by pathogens elicit defense responses in plant cells. Degradation of the pectin homogalacturonic domain results in production of oligogalacturonides, some of which have a role as plant defense elicitors (Vallarino and Osorio 2012). Changes in pectin content and degree of methylesterification in young and old leaves creates the possibility of cell wall fragment signaling involvement in apple–*V. inaequalis* interaction.

The mechanism of ontogenic resistance is not known. The identification of factors rendering older leaves resistant to *V. inaequalis* is required, as it might help design more efficient breeding programs as well as to aim specific processes in apple tree chemical protection or a plant defense against the fungus. Differences in transcriptomes between resistant old and susceptible young leaves in respect to ontogenic resistance were analyzed by Gusberti *et al.* (2013). Five candidate genes were identified whose expression variations may be related to ontogenic resistance; their involvement in a specific cellular process has yet to be identified. Our results confirmed the small role of apple cuticle in ontogenic resistance. We showed here that the formation of specific cuticular structures in apple epidermal cells did not coincide with ontogenic resistance onset. Chemical composition analysis of

apple leaf cuticle did not reveal any significant change during leaf development that might be responsible for the onset of ontogenic resistance. The role of the cuticle as a mechanical barrier in ontogenic resistance of apple to the scab is considered as improbable also by other authors because the fungus development becomes delayed after the cuticle penetration (Valsangiacomo 1988). Conditions of subcuticular environment are suggested to be involved in ontogenic resistance (MacHardy 1996). These may include sub-cuticular pH and a strengthened cell wall. In our experiments, the onset of ontogenic resistance coincided with the cessation of cell expansion and the termination of the cell shaping process. Cell shape formation requires specific changes of the cell wall polysaccharide matrix, which allows cell wall re-modelling during growth. For example, expanding cell walls have lower pH, which allows cell wall components to loosen during cell wall expansion (Hocq *et al.* 2017). Indeed, we were able to detect qualitative changes of cell walls composition in expanding and mature apple leaves. Resistant old leaves contained a higher homogalacturonan content, which also displayed a lower degree of methylesterification. Cell wall polysaccharides are probably the major carbon source for *V. inaequalis* (Fothergill and Ashcroft 1955, MacHardy 1996). *Venturia inaequalis* mycelium develops well in young leaves with rapidly expanding cells, which have an increased cell wall extensibility. Since pectin-degrading enzymes are used by the fungus during the infection (Valsangiacomo and Gessler 1992, Kollar 1998), pectin susceptibility to fungal degradation enzymes may represent an important factor for fungal growth. Highly cross-linked pectin may be much less favorable as a carbon source for *V. inaequalis*. It has been suggested that the fifth and older leaves resistant to the infection inhibit the growth of the fungus rather than kill it (MacHardy 1996, Li and Xu 2002), which supports the hypothesis of limited nutrition to mycelium. It has been frequently observed that due to the loss of ontogenic resistance in old senescing leaves, the growth of the fungus can be restored at the end of the growing season (Kollar 1996, MacHardy 1996. The restored mycelium growth may be explained by cell wall re-modeling in senescing leaves. Quantitative and qualitative changes in galacturonic acid content in old leaves also bring forth the possibility that cell wall fragment signaling functions in apple leaf response to *V. inaequalis* infection.

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