

SEKCIJA 2: BIOTSKE INTERAKCIJE
SESSION 2: BIOTIC INTERACTIONS

Plant-insect interactions: the induction of defence mechanisms

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Abstract

Herbivory by feeding arthropods such as insects elicits defence responses in the infested plants, including the emission of volatile organic compounds (VOCs) that can serve as indirect defence signals. In the elicitation of defence responses, the individual contributions of plant tissue wounding on the one hand and chemical signals introduced by the insect during the feeding process on the other hand is not clear up to now. We want to dissect both parameters to understand their particular impact on the induced plant reactions.

Using the closed loop stripping technique in combination with GC-MS analysis we identified and quantified the induced and emitted VOCs (Donath & Boland, 1995).

To mimic the herbivore-caused tissue damage as close as possible, a mechanical caterpillar (MecWorm) has been designed. MecWorm resembles herbivory in terms of a similar physical appearance and a long lasting wounding period on defined leaf areas. This mode of treatment was sufficient to induce the emission of a VOC blend qualitatively similar to that as known from real herbivore feeding, although there were significant quantitative differences for a number of certain compounds (Table 1; Mithöfer et al., 2005).

Table 1: VOCs emitted from Lima bean leaves upon different treatments.

Compound	<i>S. littoralis</i>	<i>C. hortensis</i>	MecWorm
Relative amount of VOCs (A_{VOC}/A_{IS})			
Octen-3-ol	0.09 (0.06-0.19) ^a	0.03 (0.01-0.05) ^b	0.37 (0.17-0.69) ^c
(Z)-3-Hexenyl acetate	0.47 (0.14-1.37)	0.16 (0.07-0.41) ^b	0.43 (0.34-0.59)
β -Ocimene	0.65 (0.16-1.42)	0.49 (0.01-0.67)	0.28 (0.09-1.21)
Linalool	0.37 (0.19-0.63)	0.37 (0.10-0.78)	0.19 (0.11-0.47) ^c
DMNT	0.51 (0.21-1.29)	0.36 (0.14-0.88)	0.31 (0.07-0.47) ^c
C ₁₀ H ₁₄	0.27 (0.08-0.48) ^a	0.05 (0.01-0.07)	0.08 (0.03-0.26)
MeSA	0.01 (0.01-0.03)	0.13 (0.07-0.59)	0.03 (0.01-0.09)
C ₁₀ H ₁₆ O	0.89 (0.28-1.92)	0.26 (0.05-0.32)	0.27 (0.07-0.88) ^c
Indole	0.00 (n.d.-0.28)	n.d. ^d	n.d. ^d
β -Caryophyllene	0.07 (0.03-0.17) ^a	tr ^e	0.02 (n.d.-0.06) ^c
Nerolidol	0.03 (0.01-0.07) ^a	tr ^e	n.d. ^d
TMTT	0.12 (0.05-0.21)	0.26 (0.02-0.67)	0.03 (0.01-0.07) ^c

Moreover, both the duration and the area that has been mechanically damaged, contribute to the induction of the whole volatile response. Based on those two parameters, time and intensity, which can replace each other to some extent, a damage level could be defined. That damage level exhibits a close linear relationship with the accumulation of fatty acid-derived volatiles and monoterpenes while other terpenoid volatiles and methyl salicylate respond in a non-linear manner.

In *Arabidopsis thaliana*, microarray technique was used to investigate gene regulation processes on the transcript level upon insect feeding and MecWorm treatment, respectively. Based on the whole genome background, significant changes, about 5700 genes, in transcript level have been found locally as well as systemically in both cases. Among these genes, 4100 are regulated identically, independent on the presence of chemical components from the insect. In contrast, the regulation of about 3200 genes showed that those signal compounds are involved in the systemic responses.

The results that will be presented and discussed suggest that the impact of mechanical wounding on the induction of defence responses during herbivore feeding was strongly underestimated up to now.

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Adaptation of Colorado potato beetle larvae to potato defence response at the level of digestive cysteine proteases

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Introduction

Colorado potato beetle (*Leptinotarsa decemlineata* Say, CPB) is a major pest of potato in many areas of the world. It rapidly acquires resistance to pesticides, so there is a need for alternative means of control. One possible approach is to disrupt insect protein digestion by overexpressing protease inhibitors (PIs), which negatively impact insect physiological status (Wolfson and Murdock, 1987). However, in many cases insects can adapt to the challenge of PIs by quantitative and qualitative remodelling their digestive protease complement. Protease inhibitors form part of the plant defence in response to insect attack (Green and Ryan, 1972). Cysteine proteases that are insensitive to inhibitors are produced in larval guts after feeding CPB larvae on potato leaves containing high levels of endogenous PIs (Bolter and Jongsma, 1995). Previously, three cysteine proteases (termed intestains) were purified from adapted guts, with different substrate specificities and inhibition profiles. One of the isolated intestains was also able to inactivate one of the cystatin inhibitors by cleaving off its N-terminus (Gruden et al., 2003). cDNA sequences coding for cysteine proteases were isolated and classified under three intestain groups: A, B and C (Gruden et al., 2004). Intestains A and C were induced during the adaptation process of CPB larvae to high levels of potato defence PIs. Intestains A, B and C differ in some specific structural characteristics in the inhibitor binding sites that could influence enzyme-inhibitor interaction. The aim of the current study was to analyse new intestain cDNAs isolated from adapted guts. A combination of the pattern of gene expression in response to inhibitors and protein modelling of new intestains was used to understand the molecular basis of the resistance to proteinase inhibitors in Colorado potato beetle.

Materials and methods

Defence response (including PIs) in potato plants was induced by methyl jasmonate (MeJA). CPB larvae were reared on MeJA-induced or control potato leaves for short or long periods of time. To simulate field conditions larvae were fed on the whole set of potato plants in growth chamber. Midguts were isolated from control and MeJA-adapted larvae for mRNA isolation. We constructed two subtractive cDNA libraries, one representing up- and the other down-regulated genes in MeJA-adapted midguts. Full-length cDNAs of new intestain cDNAs were amplified from MeJA-adapted midgut cDNA library. Expression of intestain genes during the larvae adaptation was analyzed using microarray and real-time PCR approach. Relative number of intestain gene copies in CPB genome was calculated from the reference material standard curve of real-time PCR assay. The structure of human cathepsin L (HICF) was used to model intestain D and E. Putative enzyme-inhibitor interactions were studied by superimposing molecular models of intestains on enzyme-inhibitor complexes of papain-stefin B (1STF) and cathepsin L-p41 (HICF).

Results and discussion

From the subtractive cDNA libraries of larvae midgut, we isolated three partial intestain-like cDNA sequences. Furthermore, 25 cDNA sequences were obtained that were highly similar to these three partial cDNA sequences and were classified under two new

intestain groups: D and E. Intestains D and E share 60% identical amino acid residues and are most identical to intestains B and A, respectively (ca. 70 % identity). Both groups of intestains are 30-52 % identical to cathepsin-L like proteases (digestive and non-digestive) from insects and other arthropods and mammalian cathepsins L, K, S and P.

Homology modelling predicts that intestains D and E follow the general fold of papain-like proteases. Unlike other papain-like enzymes, intestains D and E have the insertion of Glu64a (papain numbering) in front of the conserved Gly65 which is a part of the S1 substrate binding site (Turk et al., 1998). Superimposing of the intestain D and E models to the papain-stefin B complex reveals steric clashes of Glu64a with the inhibitor N-terminus. The other possible outcome of such structural change is that the flexible inhibitor N-terminus could adapt its conformation and shift inwards the active side cleft. In this case, the inhibitor N-terminus would come in the proximity of the catalytic residues and would therefore become exposed to the proteolytic cleavage. No major structural characteristics of intestains D and E were observed in the binding sites of tyroprolin type inhibitors that would influence the enzyme-inhibitor interaction. The amino acid residue at position 205, important for substrate specificity or inhibitor binding (Turk et al., 1998), is atypical in intestains D and E. Intestains D have Asp205 which is similar to Glu in cathepsin B (Bromme et al., 1994) by its positively charged side chain and intestains E have Met205 with hydrophobic side chain as cathepsin L. This leads to a prediction that intestains D and E would differ in their substrate specificities. Intestains E would show cathepsin L specificity and would preferentially hydrolyse substrates with hydrophobic residue in the P2 position while intestains D would favour binding a charged basic residue in the P2 position in a similar manner as cathepsin B.

Intestains D were down-regulated approximately twofold 4 h after the beginning of larvae feeding on MeJA-induced potato leaves and up-regulated twofold in the long-term adaptation, which is consistent with the expression of intestains A and C (Gruden et al., 2004). Expression of intestain E genes, similar as expression of intestain B genes (Gruden et al., 2004), remains at approximately the same level in short- or long- term adaptation to potato protease inhibitors induced by MeJA and insect wounding. Feeding of larvae in the field simulated conditions provokes less intensive adaptation than MeJA-induced plant defence.

The number of intestain E genes in CPB genome is 30-fold higher than the number of intestain D genes. High number of intestain E genes indicates the important role of intestains E in the proteolytic capacity of CPB midgut and represents a genetic reservoir of different protease isoforms presumably involved in the CPB adaptation to potato defence response.

Conclusions

The composition of digestive proteases in Colorado potato beetle gut changes upon feeding on high levels of potato defence PIs in such a way that enzymes resistant to the inhibitors are produced to compensate for the loss of activity of sensitive proteases. Most of them are induced twofold during the CPB adaptation to potato defence response. They differ in some specific structural characteristics in the inhibitor binding sites that could influence enzyme-inhibitor interaction and thus provide different mechanisms of resistance to PIs. Only when we understand the regulation and structural characteristics of the full complement of insect digestive proteases we will be able to effectively target them via protease inhibition as a pest control management.

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Analysis of gene expression in phytoplasma infected grapevine

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Introduction

Phytoplasmas are plant intracellular pathogens, living and propagating in floem cells. On *Vitis vinifera* L. they cause a group of diseases called grapevine yellows (GY). Typical symptoms are yellowing and downward curling of leaf veins and lamina, sometimes stunted shoots, uneven or total lack of lignification of canes, flower abortion or berry withering. Many of the symptoms are results of changes in sugar metabolism caused by the presence of phytoplasmas in floem (Boudon-Padieu, 2003; Lee et al., 2000).

Two main GY phytoplasmas are present in Europe: Bois Noire (BN, Stolbur group) and Flavescence Dorée (FD, Elm Yellows group). The latter is causing great economic damage and is recognized by the European and Mediterranean Plant Protection Organization (EPPO) as a quarantine pest in Europe. The facts that phytoplasmas are among the last group of plant pathogens recognized so far and that they cannot be cultivated in host-free systems result in a very poor understanding of GY pathogenesis. Our research will focus on the interaction of FD and BN phytoplasma with grapevine using real-time PCR and DNA microarrays.

Materials and methods

Plant material for RNA extraction (central leaf midribs) was collected and frozen in liquid nitrogen in the field. Samples were collected from cultivars Chardonnay, Prosecco and Barbera from healthy, infected and “recovered” plants. RNA was extracted, treated with DNase, quantified and reverse transcribed to cDNA. Luciferase RNA was added prior to reverse transcription.

Three genes were selected as marker genes for the disease status: Alcohol dehydrogenase I (Adh I), Sucrose synthase (Ssh) and Heat shock protein 70 (Hsp70) (Brzin, 2004; Cargianale et al., 2004). Amplicons for real-time PCR for these three genes were designed. Relative expression values were analysed in all samples using real-time PCR (SYBR[®] Green chemistry). Relative quantification approach was used (Pfaffl, 2001) with 18S rRNA and Cytochrom oxydase (COX) as internal controls. Amplicon that detects luciferase was used to estimate whether reverse transcription of each sample was successful. All samples were also tested for phytoplasma infection using specific TaqMan[®] MGB detection system which was also developed for the purpose of this study.

T-test was used to evaluate whether expression values of marker genes differentiated between disease states. Samples were then clustered based on expression values of AdhI, Ssh and Hsp70 using linear discriminant analysis (LDA) and hierarchical clustering. Based on these results pools of two to three original RNA samples were prepared for microarray hybridizations (70mer oligonucleotide grapevine microarrays, Grape Genome Oligo Set V1.0, Operon).

Results and discussion

T-test on all Chardonnay samples showed that AdhI and Ssh differentiated between healthy and infected samples (p -value < 0.05). LDA results were visualised in two-dimensional plot (Figure 1). Samples grouped according to their disease status (healthy were distinguished from infected) and cultivar. Even within healthy samples (of e.g. cv. Chardonnay) several subgroups could be distinguished. This showed that plants with the same disease status in the same vineyard can be in slightly different physiological states which can be detected on the level of gene expression. RNA samples that grouped closely together in LDA plot were pooled for microarray hybridisations. With this approach samples that had similar expression profiles were pooled together. Several pools of

healthy and diseased plants were thus created for co-hybridization with reference pool RNA on microarrays. In this way we will be able to distinguish genes that are differentially expressed as a result of different physiological state of plants from genes that are differentially expressed as the consequence of infection with phytoplasma.

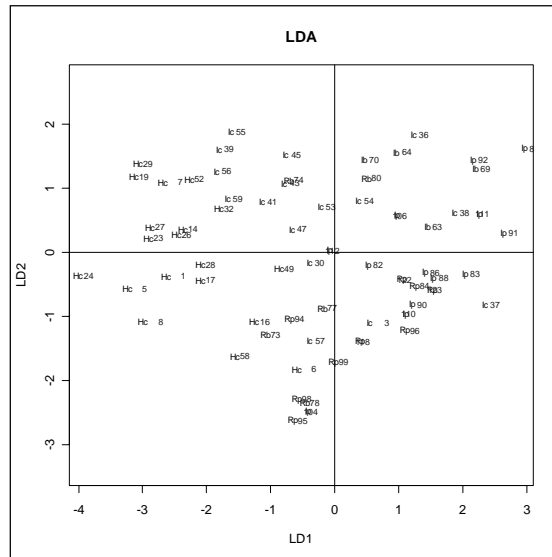


Figure 1: Graphic representation of the LDA results. Linear discriminant components (LD1 and LD2) for each sample are calculated from sample's expression profile of AdhI, Ssh and Hsp70 genes (H-healthy, I-infected, R-“recovered”, c-cv. Chardonnay, p-cv. Prosecco, b-cv. Barbera)

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Possible role of carbon metabolism alterations in development of symptoms in potato infected with PVY^{NTN} / PVY^N

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Introduction

Virus-infected plants typically develop symptoms which include stunting, leaf distortion, dark greening and chlorosis. Physiological changes include alterations in the levels and / or partitioning of different metabolites. Sugars have been reported to accumulate in photosynthetic source leaves of virus-infected plants and consequently have effects on pathways involved in pigmentation and hormone function. Alterations in sugar metabolism might have effect on symptoms (Love et al., 2005). Accumulation of sucrose (Herberst et al., 2000) or other soluble sugars, starch and chaloise is observed in virus inoculated leaves (Biemelt and Sonnewald, 2006). Increase in sugar levels leads to chlorosis, while neighbouring cells produce more pigment to compensate for the loss in carbon fixation, resulting in mosaic (Herbers et al., 1997).

Sensitive potato cultivars (*Solanum tuberosum* L.) infected with Potato virus Y^{NTN} (PVY^{NTN}) show chlorotic ring-spots on inoculated leaves and necrotic ring-spots on tubers. Disease is known as a potato tuber necrotic ring-spot disease (PTNRD) and is responsible for great losses in crop industry. PVY^{NTN} group of viruses is believed to have developed through recombination of PVY^N and PVY^O isolates. PVY^N isolates do not cause any or very mild symptoms on potato. Symptom development and their severity depend on the isolate of PVY, potato cultivar, environmental conditions and other factors (Boonham et al., 2002).

Our aim is to investigate early expression of genes involved in typical PTNRD symptoms development in potato. Two potato cultivars, Igor and Nadine are used, since both are known to be sensitive on PVY infection and show typical PTNRD symptoms after PVY^{NTN} infection and no symptoms after PVY^N infection.

Materials and methods

Potato plants cv. Igor and Nadine were divided in two groups; one was inoculated with PVY^{NTN} and the other with PVY^N. Inoculated leaves were harvested at three time points: 30 min, 12 h and 48 h after inoculation. A control group of plants was left for observation of symptom development. From harvested material total RNA was extracted using RNeasy Plant Mini Kit (Qiagen). RNA was transcribed into cDNA and labelled with dendrimeres of fluorescent dyes (Genisphere 3DNA Array 900 Kit). cDNAs from PVY^{NTN} and PVY^N inoculated plants from the same time point were hybridized on the same potato cDNA microarray (TIGR). Hybridized microarrays were scanned and analysed with ArryPro. Spots with low quality were excluded from further analysis and ratios of expressed genes were calculated. Using programme language R, data was normalized and differentially expressed genes were selected. Ratios of differentially expressed genes were visualized using MapMan.

Results and discussion

After inoculation with PVY^{NTN}, both cultivars showed typical necrotic ring-spots on inoculated leaves and tubers. On PVY^N inoculated leaves no symptoms or very mild chlorotic rings were observed. Preliminary microarray data analysis revealed differential expression of genes coding for the enzymes involved in carbon partitioning (Fig. 1). Genes involved in synthesis, degradation and

transport of sucrose and starch were observed in more detail. Genes in both pathways appear to be up- or down-regulated in plants inoculated with PVY^{NTN} in comparison to the response in plants inoculated with PVY^N. Genes for fructokinase in sucrose degradation pathway are in both cultivars at all three time points up-regulated. In the starch pathway, 30 min after inoculation genes coding AGPase and 4-alpha-glucanotransferase are up-regulated in both cultivars. In the sucrose pathway Nadine has a transient response. 30 min after inoculation genes coding hexokinase and sucrose-phosphate synthase are up-regulated, while after 12 h they are down-regulated. 48 h after inoculation those genes are again up-regulated.

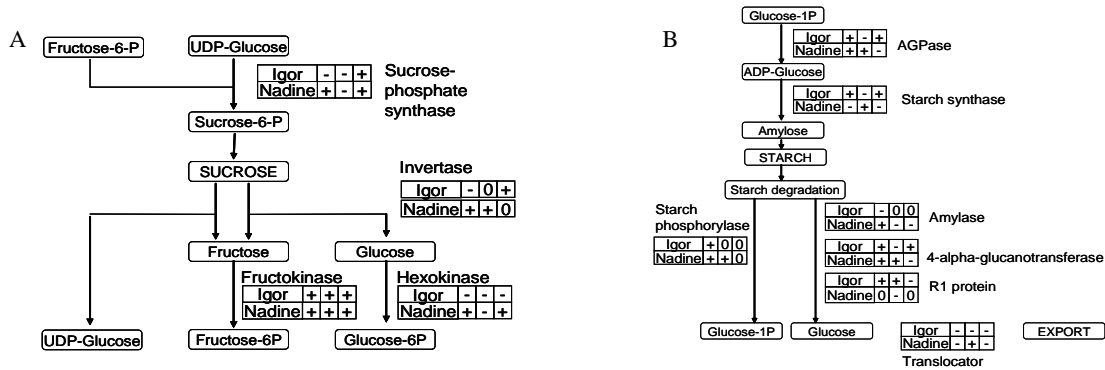


Figure 1: Metabolic pathways for sucrose (A) and starch (B). Each block responds to certain time point (1st 30 min, 2nd 12 h and 3rd 48 h), separately for cvs. Igor and Nadine. (+) stands for up-regulation and (-) stands for down-regulation of genes in PVY^{NTN} inoculated leaves in comparison to PVY^N inoculated leaves. (0) stands for no defined change in expression. Names of the enzymes quoted represent a family of the cDNA that bound on the corresponding spot.

Conclusions

Genes for the enzymes involved in sucrose and starch synthesis, degradation and transport have a possible role in symptoms development. Up-regulation of fructokinase implicates accumulation of fructose-6P in leaves inoculated with PVY^{NTN} relative to the response in plants inoculated with PVY^N (Fig.1 (A)). According to our preliminary results, cultivars have different pattern in expression of genes involved in starch and sucrose metabolism.

Acknowledgements

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Changes in the subcellular distribution of glutathione precursors during ZYMV-infection in *Cucurbita pepo* L.

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Introduction

Plants react to pathogen attack with changes in levels of antioxidants and related enzymes due to the pathogen induced oxidative stress and activation of defense genes. Especially ascorbate and glutathione are thought to play a key role in the ability of the plant to develop successful defense strategies against pathogens (Gullner et al., 2001; de Gara et al., 2003). In plants glutathione is synthesized out of its constituents cysteine, glutamate and glycine in two ATP-dependent steps. In the first step cysteine and glutamate are linked together to form γ -glutamyl-cysteine. In the second step glycine is added to form the final product glutathione. These reactions take place in plastids and the cytosol, which are therefore considered to be the main centers of glutathione synthesis (Gomez et al., 2004; Sugiyama et al., 2004; Wachter et al., 2005). Since the availability of glutathione precursors limits glutathione synthesis especially during stress situations (Kopriva and Rennenberg, 2004) the aim of this study was to investigate how the subcellular distribution of glutathione precursors correlates with the subcellular distribution of glutathione during compatible virus infection found in a previous study (Zechmann et al., 2005). Therefore immunogold labeling of cysteine, glutamate and glycine was performed on control and *Zucchini Yellow Mosaic Virus* (ZYMV)-infected *Cucurbita pepo* L. plants. The advantage of this technique over biochemical and light microscopical studies is that it visualizes the subcellular distribution of labeling, not only in whole organs, but within all cellular components at a high level of resolution in one experiment simultaneously.

Materials and methods

Two week old *Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb. (Styrian oil pumpkin) plants were mechanically inoculated with ZYMV and prepared for cytohistochemical investigations as described previously (Zechmann et al., 2005). Immunogold labeling of glutathione precursors was performed with ultrathin sections on nickel grids. Samples were blocked with bovine serum albumine in phosphate buffered saline (PBS) and then treated with the primary antibodies against cysteine, glutamate and glycine in PBS. Subsequently after a short rinse in PBS the samples were incubated with a 10 nm gold-conjugated secondary antibody. After a short wash in distilled water labeled grids were observed in a Philips CM10 TEM. Randomly photographed immunogold labeled sections of the mesophyll from older and younger leaves and meristematic root tip cells were digitized and gold particles were calculated automatically using a software package (Optimas 6.5.1) in different manually identified cell structures (mitochondria, plastids, nuclei, peroxisomes, the cytosol and vacuoles).

Results and discussion

In previous studies we found that ZYMV-infection induced a much stronger increase in glutathione contents in cells of younger leaves, especially in glutathione producing organelles like plastids and the cytosol than in older leaves (Zechmann et al., 2005). While levels of cysteine and glutamate decreased up to 54% and 52% in plastids of cells from both ZYMV-infected leaf types, levels of glycine increased between 1.5-fold to 1.3-fold in plastids and the cytosol, respectively, of ZYMV-infected younger leaf cells and decreased between 53% to 22% in plastids and the cytosol, respectively, in cells of ZYMV-infected older leaves (Fig. 1). These results indicate that low levels of glycine especially in plastids and the cytosol, as the main centers of glutathione synthesis in plants, are the limiting factors of glutathione synthesis during compatible ZYMV-infection in the older leaves. Thus leading to the observed lower increase in glutathione levels in these organs when compared to younger ones. The loss in the ability to provide enough

glutathione in the older leaves could in the long term be responsible for gradually advancing symptom development, which resulted in virus induced senescence and cell death. That the availability of glycine can limit glutathione synthesis during stress situations has also been discussed by Noctor et al., 1997. Usually the availability of cysteine and γ -glutamyl-cysteine is the limiting factor for glutathione synthesis (Kopriva and Rennenberg, 2004). But in situations when glutathione synthesis is high e.g. during the present biotic stress, high levels of glycine are required to sustain maximal rates of glutathione synthesis (Noctor et al., 1997). Decreased levels of glutathione in roots of up to 32% in mitochondria during ZYMV-infection were probably due to interruptions of glutathione-transport from the leaves since glutathione precursor levels were strongly increased or remained unchanged (Fig. 1) in glutathione producing organelles of roots from ZYMV-infected plants. Glutathione and its precursors were virtually absent in cell walls and intercellular spaces and play therefore no important role during ZYMV-attack in these cell compartments. Summing up the results of the present study indicate that glycine is the limiting factor of glutathione synthesis during ZYMV-infection in older leaves leading to a much weaker increase in glutathione levels in this organ in comparison to younger ones. Decreased levels of glutathione in ZYMV-infected roots are not due to limiting amounts of glutathione precursors.

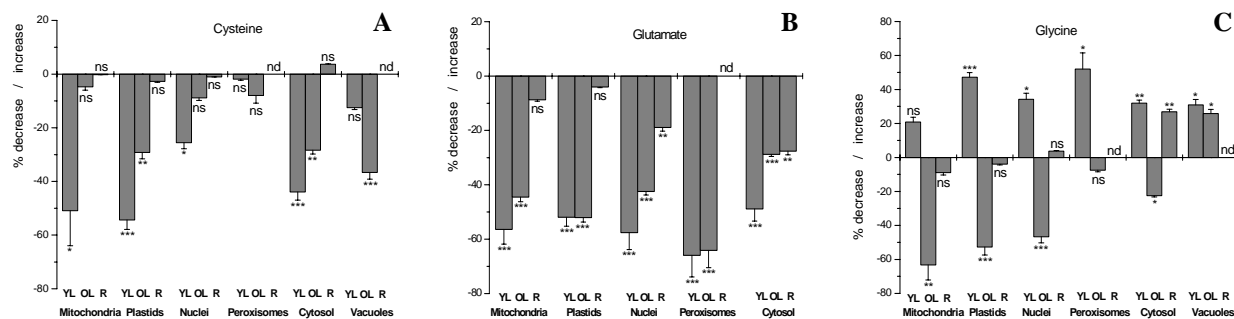


Figure 1: Values are means \pm standard errors and document the percentage of increase or decrease of the amount of gold particles bound to A) cysteine, B) glutamate (absent in vacuoles) and C) glycine in ZYMV-infected leaf mesophyll or root tip cells in comparison to the control. nd=not determined; $n > 20$ for peroxisomes and $n > 60$ for other cell structures from at least 15 different cells. Significant differences between organelles of control and organelles of ZYMV-infected cells were calculated with the Mann Whitney U-test. ns if $p > 0.05$, (*) if $p < 0.05$, (**) if $p < 0.01$, and (***) if $p < 0.001$; YL=younger leaves, OL= older leaves and R=roots.

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Isolation and characterization of intestains from Colorado potato beetle gut

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Introduction

Colorado potato beetle (*Leptinotarsa decemlineata* Say) is one of the major pests on potato (*Solanum tuberosum* L.). After insect attack a synthesis of proteinase inhibitors is initiated in plant. During digestion of plant material synthesis of digestive proteases insensitive to proteinase inhibitors is induced in larvae guts. We tried to isolate insensitive cysteine endoproteases from digestive tract, named intestains, which cleave cystatine-type model inhibitor and are inhibited by equistatin. We were particularly interested in intestains group DI (also named intestains type 3), which also cleave specific substrate *p*-Glu-Phe-Leu-*p*NA.

Materials and methods

Synthesis of proteinase inhibitors was initiated in plants with methyl jasmonate treatment and Colorado potato beetle larvae were fed with treated potato plants. Guts were extracted with extraction buffer (0,9 % NaCl, 5 % polivinil piroolidon) and proteins were isolated using acetone precipitation and gel filtration (Sephacryl S-200, Pharmacia, Sweden). Samples were concentrated with ultrafiltrator. System for affinity chromatography with equistatin as a biological active molecule was used for further cleaning and Centricon YM-10 (Amicon, USA) for concentration. Intestains were detected spectrophotometrically at A₄₀₅ using *p*-Glu-Phe-Leu-*p*NA (Sigma) as a substrate. SDS-PAGE and IEF gels (PhastSystem, Pharmacia, Sweden) were used to separate intestains. Bands from SDS-PAGE gel were excised and 2 of them were analysed with MALDI-TOF MS. Sequences were generated in program Peptidmass (<http://www.expasy.org/tools/peptide-mass.html>) and compared with database of known sequences.

Results and discussion

Acetone precipitation and gel filtration proved to be efficient for protein separation. Several systems for affinity chromatography were prepared, but binding of intestains to the affinity matrix did not occur although equistatin did inhibit the proteolytic activity. It is possible that during preparation binding sites for sepharose or intestains are changed. Alternative approach is ion-exchange chromatography which has not yet been tested for intestain isolation.

The fully degradation of human cystatin C was proven with fractions after gel filtration, while in the same incubation time only partially degradation was observed after the affinity chromatography. During ultrafiltration about 70 % of proteolytic activity was lost. Fractions obtained after the affinity chromatography were concentrated using Centricon and analysed on SDS-PAGE and IEF gels to determine molecular mass and isoelectrical point of intestains (Fig. 1, Fig. 2). Molecular mass of intestains was between 23 and 24 kDa and pI between 4 and 4,5. Bands were cut from IEF and SDS-PAGE gels, transferred to activation buffer and tested for cystatin C and *p*-Glu-Phe-Leu-*p*NA degradation. Reactivation of intestains from the gel is possible but very slow and probably incomplete. Bands from SDS-PAGE gel that showed degradation activity were analysed using MALDI-TOF MS and generated sequences represented intestains groups DI, DII, C and E (Fig.3). The most interesting is intestain DI group (intestains type 3) whose members (IntD4, IntD6 and clone 4-160-R2) were isolated in one band of SDS-PAGE gel (Fig. 1, band 2). Because of very small amount of enzymes (less than 40 µg) incorporated in gel only shorter sequences were obtained (Fig. 3, coloured). More appropriate for isolation of longer amino acid sequences would be SDS-PAGE electrophoresis with higher capacity.

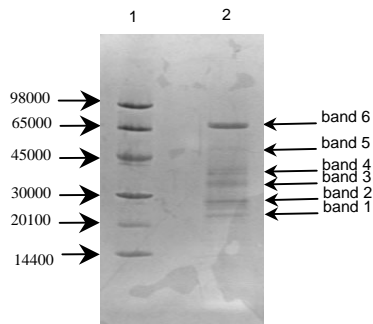


Figure 1: SDS-PAGE of concentrated eluat through Centricon.
Centricon.
Line 1: MW standard (Da), line 2: concentrated eluat.

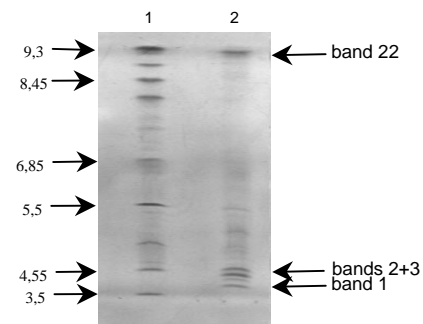


Figure 2: IEF of concentrated eluat through
Centricon.
Line 1: IEF standard, line 2: concentrated eluat.

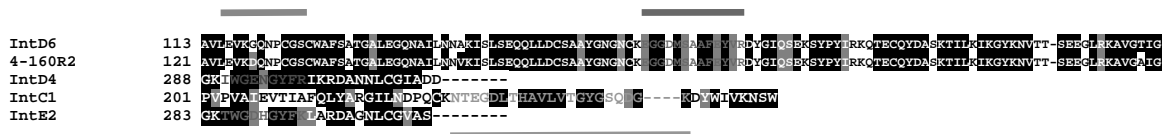


Figure 3: Alignment of amino acid sequences of digestive proteases from adapted Colorado potato beetle gut (an example).

Conclusions

In this work we have shown that concentrating is the crucial step in isolating enzymes since 70 % of proteolytic activity was lost with ultrafiltrator. MALDI-TOF MS after SDS-PAGE separation is appropriate tool for analysing amino acid sequences. Intestains group DI were isolated in one band on SDS-PAGE gel. We proved that reactivation of these enzymes from band is possible. We have also confirmed that molecular mass of intestains group D is between 23 and 24 kDa and pI between 4 and 4,5.

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Infection with fungus *Venturia inaequalis* increases the phenolic content of apple leaves

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Introduction

Phenolic compounds are involved in natural defence reactions of apple (*Malus domestica* Borkh.) against various diseases. They are toxic to pathogens and are produced and accumulate at a faster rate after infection. In apple, phenolic compounds and especially flavanols play an important role in resistance to the fungus *Venturia inaequalis* (Cooke) G. Wind. Aderh. causing apple scab (Treutter and Feucht, 1990). Chlorogenic acid, phloridzin as well as other phenolics have been shown to play an important role in host resistance to pathogen, because they inhibit the development of the fungus (Hamazu, 2006).

The aim of the study was compare the differences in containing the single phenolic compounds and total phenolic content in healthy leaves and with fungus *Venturia inaequalis* infected leaves of cultivar 'Golden Delicious'.

Materials and methods

Leaves were taken in year 2005 from the 'Golden Delicious' apple trees. Healthy and scab infected leaves were collected. From infected leaves only infected tissue with surrounding cells (1-2 mm) was taken. The extraction of the leaves followed the method described by Colarič et. al. (2006) with minor changes. The phenolic compounds were analyzed on HPLC-PDA system. The hydroxycinnamic acids (chlorogenic, p-coumaric) and the flavan 3-ols (catechin, epicatehin) were detected at 280 nm, whereas phloridzin was estimated at 350 nm. The column used was a Phenomenex Gemini C₁₈. The samples were analyzed as described by Escarpa and Gonzales (2000) with slight changes. The concentrations were expressed as mg 100 g⁻¹ dry weight or mg g⁻¹ dry weight (DW). Total phenolic content of extracts was assessed by using the Folin-Ciocalteu phenol reagent method (Singleton & Rossi, 1965). Absorption at 765 nm was measured in a UV-Vis Spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram dry weight of leaves samples. The data were analysed by using the Statgraphics Plus 4.0 program. One-way analysis of variance was used (p<0,05).

Results and discussion

The chlorogenic acid is the most widely represented among hydroxycinnamic acids. Its content ranges from 26 to 50 mg/100 g of healthy leaves, which is comparable with the results obtained by Picinelli et al. (1995), where the apple leaves contained from 11 to 113 mg/100 g leaves. The leaves infected with scab contained significantly more chlorogenic acid as healthy leaves, from 132 to 267 mg/100 g leaves. With the scab infection the content of p-coumaric acid increased. In general the content increased by 1-2.5 times compared to healthy leaves. Also, the previously published findings show that apple leaves respond to fungus infection by accumulation of phenols. Bennet and Wallsgrave (1994) found, that in some cases after the infection of apples with *Venturia inaequalis* the derivatives of chlorogenic and coumaric acid accumulated. Healthy leaves contained from 26 to 49 mg/100g DW of catechin and 75 to 162 mg/100g DW of epicatechin. Our results are comparable with those from the previous publications. We have noticed that in infected leaves higher concentrations of epicatechin and catechin were synthesised. The infected leaves of apple trees contained from two to three times more catechin and 1.5 to 3.5 times more epicatechin in comparison with healthy leaves. Accumulation of flavan-3-ols in tissues of apple trees infected by fungus *Venturia inaequalis* was also reported by Treutter and Feucht (1990) and Picinelli et al. (1995). The contents of phloridzin in healthy leaves were from 81 to 139 mg/g DW. Similar contents were

obtained by Mayr (1995) and by Leser and Treutter (2005). As it is evident from our results, at the infection with fungus *Venturia inaequalis* more phloridzin is synthesized; this was also confirmed by Leser and Treutter (2005).

The infection with fungus *Venturia inaequalis* resulted in an increase of total phenols on the infected spots of leaves. Healthy leaves contained statistically less total phenols than the scab infected leaves.

Table 1: The content of phenolic compounds (mean \pm SE in mg/100g or in mg/g (phloridzin) dry weight) and the total phenolic content (TPC in mg GAE/g) in the healthy leaves and in the infected leaves of cultivar 'Golden Delicious' at different dates.

Termin	Treatment	Chlorogenic acid	<i>p</i> -Coumaric acid	Catehin	Epicatehin	Phloridzin	TPC
2.6.	healthy	30.9 \pm 6.1a	12.4 \pm 0.4a	45.4 \pm 1.4a	75.3 \pm 2.6a	139.3 \pm 8.2a	80.2 \pm 3.9a
	scab	145.7 \pm 7.3b	23.8 \pm 1.2b	104.2 \pm 5.5b	156.8 \pm 10.8b	309.1 \pm 15.7b	158.7 \pm 7.6b
23.6.	healthy	50.7 \pm 2.9a	12.2 \pm 0.7a	49.4 \pm 3.3a	82.4 \pm 17.6a	83.3 \pm 7.6a	63.9 \pm 8.7a
	scab	193.4 \pm 29.2b	30.4 \pm 1.1b	117.6 \pm 6.7b	168.9 \pm 12.4b	285.7 \pm 12.9b	135.1 \pm 5.3b
13.7.	healthy	33.3 \pm 6.2a	12.5 \pm 0.6a	36.3 \pm 3.1a	120.8 \pm 22.3a	122.1 \pm 18.1a	64.7 \pm 5.5a
	scab	197.4 \pm 31.7b	32.8 \pm 2.3b	97.2 \pm 8.0b	309.1 \pm 18.8b	327.5 \pm 23.3b	148.9 \pm 9.0b
25.7.	healthy	35.1 \pm 7.1a	11.3 \pm 2.0a	36.1 \pm 9.0a	92.6 \pm 28.2	100.9 \pm 13.6a	76.5 \pm 7.1a
	scab	220.2 \pm 34.2b	25.8 \pm 3.1b	91.9 \pm 7.5b	233.5 \pm 47.0	201.4 \pm 22.5b	152.7 \pm 7.5b
2.8.	healthy	26.9 \pm 2.1a	13.1 \pm 2.7a	26.5 \pm 6.6	116.9 \pm 9.7a	92.6 \pm 8.3a	81.8 \pm 5.6a
	scab	132.2 \pm 34.4b	30.8 \pm 1.9b	55.7 \pm 12.4	365.5 \pm 40.8b	198.9 \pm 10.0b	153.3 \pm 9.2b
22.8.	healthy	43.4 \pm 6.2a	15.7 \pm 2.0a	37.4 \pm 4.1ba	162.1 \pm 20.8a	112.4 \pm 18.7a	68.3 \pm 5.8a
	scab	267.6 \pm 30.1b	31.2 \pm 2.9b	101.2 \pm 5.2ab	376.8 \pm 19.8b	199.7 \pm 10.1b	154.2 \pm 7.0b
12.9.	healthy	40.9 \pm 13.3a	17.2 \pm 1.1a	28.5 \pm 4.1ba	148.9 \pm 16.4a	81.7 \pm 5.5a	61.9 \pm 3.6a
	scab	194.4 \pm 28.4b	35.9 \pm 1.3b	77.8 \pm 10.0b	347.9 \pm 36.5b	153.4 \pm 28.4b	124.8 \pm 5.0b

Conclusions

On the basis of the previous investigations conducted by various authors it was expected that the leaves infected with scab would present a different phenolic pattern than the healthy leaves. These findings were confirmed by the results of our investigation. The scab infected leaves contained statistically significantly more phenolic compounds in comparison with healthy leaves.

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Sclerotia and ectomycorrhizae of *Cenococcum geophilum* of young beech trees from the Free-Air Canopy O₃ Exposure system

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Introduction

In plants exposed to ozone, carbon allocation to roots and mycorrhizae can be reduced due to decreased carbon assimilation and increased metabolism above ground. Mycorrhizal fungi are differently sensitive or tolerant to stresses in the environment, while functional compatibility of the symbiosis depends on the species and strain of the fungus and of the plant. *C. geophilum* (Cg) is one of the most frequently encountered ectomycorrhizal fungi in nature. It is a cosmopolitan, has an extremely wide host and habitat range and is recognized as a pioneer species in different soil substrates and extreme ecosystems (LoBuglio 1999). The persistence of its sclerotia in soil for several years is a reservoir of fungal inoculum for new seedling re-establishment and can provide means of overcoming hostile environmental periods. These sclerotia have also been reported to represent an important source of assimilated carbon from plant species (ibid.). The occurrence of Cg mycorrhizae or sclerotia have been among first ectomycorrhizal types suggested for application as bioindicators of stress (Kowalski 1987); however in mycorrhizal inoculum potential studies their abundance was equally high in polluted and non-polluted substrates (Al Sayegh Petkovšek and Kraigher 1999) or forest research plots (Kraigher et al. 1996). The applicability of the occurrence of Cg ectomycorrhizae or its sclerotia was tested in as possible bioindicators of ozone induced stress in beech plants.

Material and methods

The occurrence of *C. geophilum* sclerotia and mycorrhizae of adult and young beech trees grown in containers, exposed either to ambient or doubled tropospheric ozone fumigation (from the Free Air Canopy O₃ Exposure system in Kranzberg Forest, near Freising, Germany, <http://www.casiroz.de>) during two consecutive growing seasons was assessed. Beech seedlings were planted into 20 containers (6 plants per 30 l container) filled with soils from the plot and exposed for two vegetation periods in the sun part of crowns (on towers) or in the shadow (at ground level). The seedlings were watered by hand and kept moist throughout the experiment. Whole plants from containers were excavated at the end of experiment in October 2004. At the end of the second vegetation period twelve 2x2x2 cm³ substrate cubes at every 2 cm depth were taken in four replicates per sun-exposed container, from which five were from the fumigated 2xO₃ and three from the control 1xO₃ fumigation system. Sclerotia of *Cenococcum geophilum* were counted under stereomicroscope in 12 depth layers and their occurrence analysed by SigmaStat3.1 and presented by SigmaPlot9.0. Two-way analysis of variance including Fisher LSD method for all pairwise multiple comparison procedures was applied per treatment and depth layer for number of sclerotia.

Results and discussion

Ectomycorrhizae of *C. geophilum* in the adult beech stand were significantly higher under the fumigated trees, while no significance was established in the physiology of the young beech plants. The abundance of sclerotia was significantly higher in the 2 to 8 cm depth layers of the fumigated containers (Figure 1). Since the abundance of sclerotia occurred before any other observable changes in plants (Herbinger et al 2004), these easily observable structures might be applied as early bioindicators of stress.

Occurrence of sclerotia of *Cenococcum geophilum* in containers

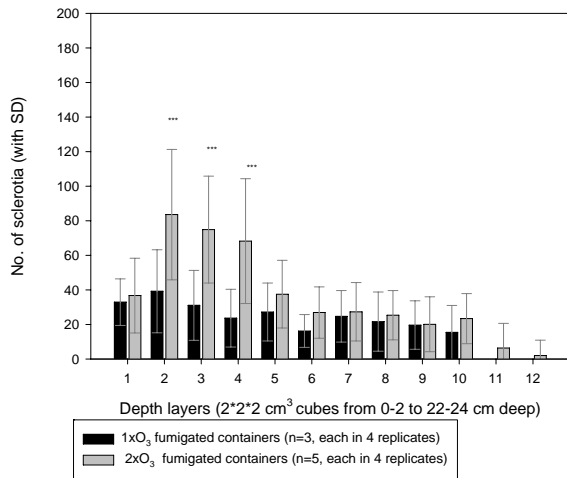


Figure 1: Number of sclerotia of *Cenococcum geophilum* in 2 cm substrate layers per sun exposed container in ambient (three containers) and double-O₃-fumigated experimental set-up (five containers) in Kranzberg forest (quantification done in four replicates per depth layer per container, means with SD; *** marks statistically significant difference (P<0,001) as tested by Fisher LSD method)

Acknowledgements:

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Contribution of gene 2A from *Grapevine fanleaf virus* RNA2 to genetic variability

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Introduction

Grapevine fanleaf virus (GFLV) causes a severe degeneration of grapevines (*Vitis* L.) with worldwide economical impact (Andret-Link et al., 2004). This virus is transmitted by grafting and by the nematode *Xiphinema index*. Current control strategies of GFLV are of limited efficacy. Therefore, new control measures, such as cross-protection and production of resistant transgenic rootstocks, are emerging (Andret-Link et al., 2004). Studies on the variability dynamics of naturally occurring GFLV populations are needed to evaluate potential environmental risks associated to these new control strategies before their implantation. Nine GFLV infected grapevines (cv. Volovnik) from a vineyard site in South-Western Slovenia were chosen for analysis of the variability of the 2A gene of GFLV RNA2. The number and combination of restrictotypes determined by IC-RT-PCR-RFLP varied depending on the GFLV isolate tested. Analysis of the sequence of the 2A gene of nine GFLV isolates indicated the occurrence of mixed infection and recombination. This is the first report on the variability of the 2A gene of GFLV-RNA2.

Material and methods

Grapevine samples were collected in a vineyard in South-Western Slovenia. Typical GFLV symptoms were recorded in the field. Leaf samples were collected and tested for the presence of GFLV using a double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) with a GFLV-specific polyclonal antiserum (Bioreba). ELISA-positive samples were used to amplify the 2A gene of GFLV RNA2, which encodes for a putative homing protein, by immunocapture (IC)-reverse transcription (RT)-polymerase chain reaction (PCR) assay using GFLV-specific antiserum (Bioreba) for IC, a specific RNA2 primer for RT, and primers flanking the 2A gene for PCR. Amplified and purified 2A gene products were then digested with *Sly*I. The digested DNA products were analyzed by electrophoresis on 2% agarose gels. Different restriction fragment length polymorphisms (RFLP) were detected by analyzing *Sly*I profiles with the demo version of Gel-Pro Analyzer software (Media Cybernetics). For sequencing, the whole RNA2 was amplified by IC-RT-PCR using a specifically designed primer pair, cloned into pGEMTeasy following the manufacturer instructions (Promega), and clones were sequenced with appropriate primers. The VectorNTI-7 package (InforMax, Inc.) was used for sequence analysis and for determining in-silico RFLP. ClustalX (Thompson et al., 1997) was used for building neighbour joining trees with bootstrap values and the RDP2 program (Martin and Rybicki, 2000) was used to detect potential recombination events.

Results and discussion

The *Sly*I RFLP pattern of nine GFLV isolates varied depending on the host plant. Three different restrictotypes (H, B, and I) were found. While some of the plants were infected with only one of the 2A restrictotypes (Vol2/52 and Vol2/57), others were infected with different combinations of two (Vol2/47, Vol2/49, Vol2/50, Vol2/51 and Vol2/55) or all three 2A restrictotypes (Vol2/45 and vol2/54). In order to confirm these results and to check for possible recombination events, we further cloned and sequenced the 2A gene from each GFLV isolate. The phylogenetic tree, corresponding to the alignment of the 2A gene from all the clones, indicated

the presence of two well differentiated (sequence diversity of app. 12%) groups (1, 2), and one sub-group (3) that is more similar to group 2 (sequence diversity of app. 4-5%) than to group 1 (sequence diversity of app. 7-8%) (Figure 1A). Intra-group sequence diversity was in all cases $\leq 1.3\%$. These data suggested a possible recombination event in the 2A gene between members of groups 1 and 2, which might have resulted in the emergence of sub-group 3. Recombination between members of group 1 and 2 was detected in nt position 225 of 2A gene by using the RDP2 program (Figure 1B). Some plants (Vol2/51, Vol2/54, Vol2/55 and Vol2/57) were co-infected with representatives of groups 1 and 2. The in-silico *StyI* RFLP calculations based on the 2A sequences showed a good correlation with the experimental data. The same three experimental RFLPs (H, B and I) were detected with a perfect correspondence with variants in groups 1, 2 and 3, respectively (Figure 1A). Furthermore, we did not find any correlation between GFLV disease symptoms and 2A restrictotype or combination of 2A restrictotypes.

In conclusion, we studied the variability of the 2A gene of GFLV isolates infecting a natural vineyard in Slovenia. We found two distinct GFLV variant groups co-existing together with a third sub-group, which results from a recombination event between representatives from the two former groups. A good correlation between the estimated and real variability by means of RFLP analysis was obtained by sequencing the 2A gene. This study will be extended to the entire GFLV RNA2, including the 2B and 2C genes.

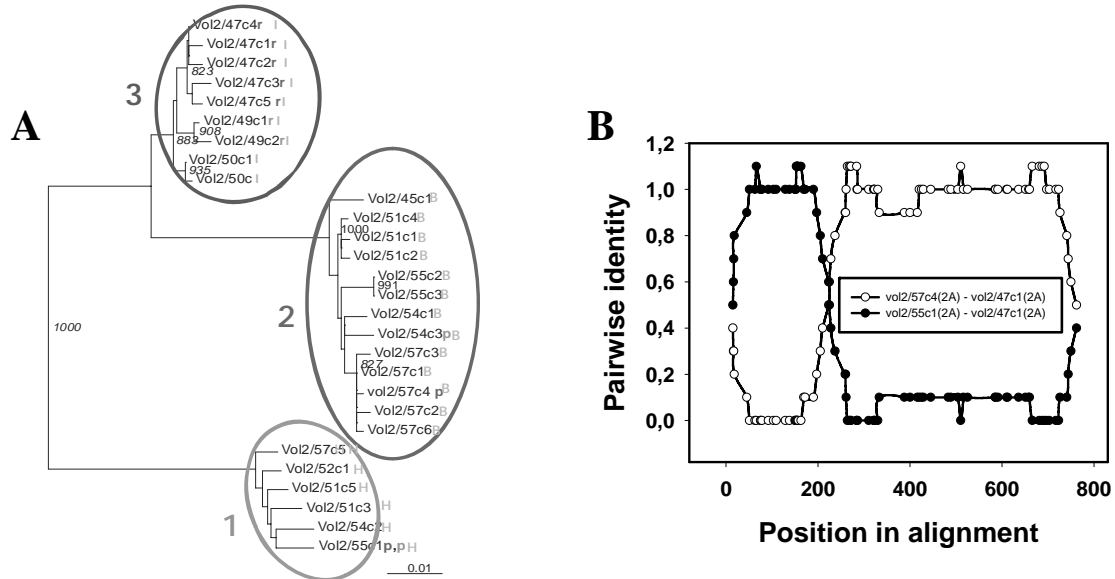


Figure 1: A) Phylogenetic tree corresponding to the alignment of the 2A gene sequences. The in-silico RFLP data is shown beside each clone. Vol2/47-vol2/57 are the grapevine hosts of GFLV isolates and c1 to c5 are the clones sequenced per isolate. Recombinants are marked with r and potential parents with p. Group 1 (yellow), group 2 (blue) and group 3 (green). B) Recombination in 2A gene of vol2/47c1 (group 3) by using the RDP2 program with vol2/55c1 (group 1) and vol2/57c4 (group 2) as parents.

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Analysis of posttranscriptional gene silencing based defence mechanism in transgenic plants of *Solanum tuberosum* L. cv. Igor

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Introduction

Necrotic isolates of Potato virus Y (PVY^{NTN}), which cause potato tuber necrotic ringspot disease (PTNRD), causes disease problems in many potato cultivars (Kus, 1994). Genetic engineering allows introduction of pathogen-derived resistance into economically important cultivars. As one of the possible mechanism of transgenic resistance in these cases a post-transcriptional RNA-mediated gene silencing response was proposed. The gene silencing mechanism is based on a nucleic acid homology. Gene silencing plays important roles in diverse biological processes including developmental regulation and antiviral defence. The key features of RNA silencing include the production of 21-25 nucleotides small RNAs by Dicer from dsRNA and the formation of Argonaut (AGO)-containing RNA-induced silencing complexes (RISCs) that directly carry out gene silencing at the transcriptional or post-transcriptional level (Qi and Hannon, 2005). New powerful technologies, which are more sensitive, are possible to detect the expression of genes involved in different genetic pathways and compare the differences in expression between the different phenotypes (sensitive / resistant) of plants.

In our study we analyzed the genetically transformed plants of potato (*Solanum tuberosum* L.) cv. Igor. The transgenic lines were transformed with a gene of coat protein of PVY^{NTN} virus and tested against PVY^{NTN}. The modern technologies that enable systemic approach were used to investigate the changes in gene expression in different sensitive and resistant transgenic lines after the inoculation with PVY^{NTN}.

Methods

In our study we analyzed four genetically transformed lines (line 16, 29, 34 and 35) of potato cv. Igor. The transgenic lines were transformed with a gene of coat protein of PVY^{NTN} virus. Lines 34 and 35 exhibited resistance to PVY^{NTN} infection while lines 16 and 29 remained sensitive to PVY^{NTN} infection (Stanič-Racman, 2002).

Four-week-old transgenic plants *S. tuberosum* cv. Igor where grown in soil in a growth chamber under controlled light and temperature conditions. Three fully expanded leaves were mechanically inoculated with PVY^{NTN} strain freshly extracted from *Nicotiana tabacum* cv. Samsun. Plant material was collected four, eight, twelve and twenty-four hours post-infection. Mock inoculated plants were collected at the same time points and used as control. Every experiment was repeated three times. With a real-time PCR method we analysed expression of the genes, which are known to be involved in gene silencing for all the collected samples. Potato cDNA microarrays were used to investigate the changes in gene expression in sensitive and resistant transgenic lines in a more systemic way. In the microarray study the samples harvest 8 and 12 hours after the inoculation were included. Quality control of microarray data was performed using image software ArrayPro Analyzer and tools in Bioconductor packages of R software. The filtered data were normalized with »LOWESS« (locally weighted linear regression) with 10 iterations (Quackenbush, 2002) and post-filtered (Kralj, 2006). The final data were analyzes with a statistic test ANOVA (ANalysis Of VAriance between groups). The final data (significantly expressed genes) were representing as heatmaps (software MeV 3) (figure 1).

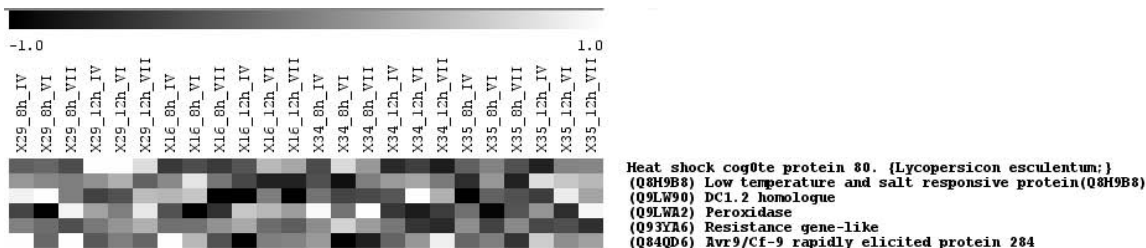


Figure 1: Heatmap of significant expressed genes showing log expression ratios between virus inoculated and mock inoculated samples. 29 - sensitive transgenic line 29, 16 - sensitive transgenic line 16, 34 - resistant transgenic line 34 and 35 - resistant transgenic line 35: transgenic lines; 8h or 12h: hours after the inoculation; IV, VI or VII: biological repeats.

Results and discussion

Some of the transgenic potato lines included in our study showed resistance and the gene silencing or RNA mediated resistance for transgenic resistance was proposed (Stanič-Racman, 2002). The presence of siRNA molecules in resistant lines was confirmed, which show the presence of gene silencing mechanism. Recently introduced microarray technology was used to investigate the changes in gene expression in different sensitive and resistant transgenic lines. The different response of different phenotypes was revealed. The analysis of gene expression profiling shows that the other defence mechanisms including gene silencing are also involved in resistance mechanism of resistant transgenic lines. With a real-time PCR method we detected the genes, which are known to be involved in gene silencing. Results show the variable expression of genes between the transgenic lines.

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The phylogenetic studies of Slovenian isolate Potato virus Y^{NTN}-NIB (PVY^{NTN}-NIB)

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Introduction

Potato virus Y (PVY) (*Potyvirus*, *Potyviridae*) is one of the most common viruses found in potato. Necrotic isolates of Potato virus Y (PVY^{NTN} (necrotic strain tuber necrosis), which cause potato tuber necrotic ringspot disease (PTNRD), have become widespread throughout Europe. It causes disease problems in many potato cultivars that were previously not affected by other strains of PVY. PVY has been found to be genetically variable. Therefore, it is interesting object to study the aspect of genome evolution in relation to its biological properties. In the course of our research work we have determined a sequence of genomic RNA of Slovenian isolate PVY^{NTN}-NIB. A comparison of genome sequence between PVY^{NTN}-NIB and the published sequences of other PVY isolates was performed.

Material and methods

Total RNAs were extracted from leaf samples of tobacco cv. Samsun infected plants, and cDNA was amplified using the Roche 'Titan One Tube RT-PCR System. Two methods (using either cloned sequences cloned into the pGEM[®]-T Easy (Promega) or RT-PCR products) were used to prepare PVY genome for sequencing. For both methods, PVY-specific oligonucleotides used for amplification and sequencing. Sequencing reactions were done on both strands. Amplified cDNA fragments were sequenced using an Applied Biosystems DNA sequencing kit: ABI PRISM[®] BigDye[™] Terminator Cycle Sequencing Ready Reaction v2.0. Sequencing products were analyzed in ABI PRISM[™] 377 or ABI PRISM[™] 310 automated DNA Sequencer (Applied Biosystems). Contiguous sequence across the PVY^{NTN}-NIB genome was derived from sequences from the overlapping RT-PCR products. The overlapping sequence 'contigs' were assembled with the BioEdit software package. Phylogenetic trees based on sequence alignments were made using the TreeView software.

Results and discussion

A sequence of 9647 nucleotides was determined for the genomic RNA of PVY^{NTN}-NIB (Accession number AJ585342). The sequence contains one large open reading frame of 3061 amino acids (Accession number CAE51230), a noncoding region of 152 nucleotides at the 5' end and a 312- nucleotides 3' nontranslated region. The nucleotides sequence and the predicted amino acids sequence of the polyprotein of PVY^{NTN}-NIB were analyzed pairwise with the available complete sequences of published PVY genomes (Thole *et al.*, 1993; Singh and Singh, 1996; Jakab *et al.* 1997, Nie and Singh, 2003). The overall relationship between PVY^{NTN}-NIB and PVY-H shows a nucleotide sequence identity of 99.2% and an amino acid sequence identity of 99.0%. The lowest degree of homology was between PVY^{NTN}-NIB and PVY^O-139. The genome sequence of Slovenian isolate showed that it had a recombinant genome comprising large sections of sequence that are characteristic of N and O sub-group isolates. Several recombination events O and N-type of genome were found in the nucleotide genome sequence of isolate PVY^{NTN}-NIB (recombination site within P3 gene, within NIa gene and within CP gene (figure 1)). In the amino acid genome sequence of PVY^{NTN}-NIB isolate two recombination sites were found (recombination site within P3 protein and within NIa protein (figure 2)).

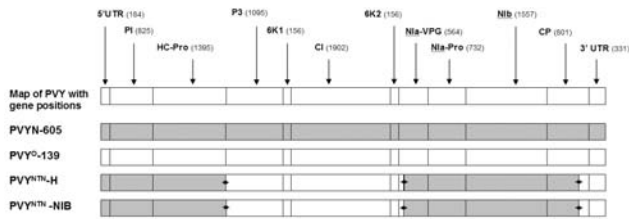


Figure 1: Nucleotide sequence maps of complete PVY genomes showing main sequence types and recombination points.

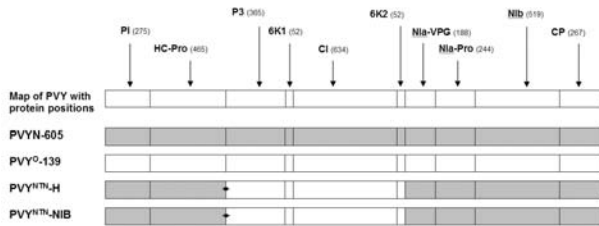


Figure 2: Amino acid sequence maps of complete PVY genomes showing main sequence types and recombination points

Conclusions

PVY has been found to be genetically variable. Several studies report on the effect of genome changes and evolution in relation to its biological properties. In the course of our research work we have reported a sequence of genomic RNA of Potato virus Y^{NTN}-NIB. A comparison of genome sequence between PVY^{NTN}-NIB and the published sequences of other PVY isolates was performed. Slovenian isolate PVY^{NTN}-NIB has shown to be almost identical with PVY^{NTN}-H isolate (Thole *et al.*, 1993). The PVY^{NTN}-H has a recombinant genome; part of genome is similar to PVY^N (Jakab *et al*, 1997) and the other part to PVY^O-139 (Singh and Singh, 1996). The phylogenetic studies had shown the same result for Slovenian isolate with an insignificant difference in the exact position of the change between type N and O type patterns.

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Peroxidases in very early responses of different potato cultivars to PVY^{NTN} infection

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Introduction

Plant peroxidases (POD) are ubiquitous enzymes that play an integral role in plant physiology, including plant-pathogen interactions (Hiraga et al., 2001). Potato virus Y^{NTN} (PVY^{NTN}) causes severe potato tuber ring necrotic disease and constitutes a good model for studying plant-pathogen interactions. In order to examine how POD are involved in the very early response of potato to infection with PVY^{NTN}, they were investigated in four cultivars of potato (*Solanum tuberosum* L.) infected with PVY^{NTN}: in very susceptible cv. Igor, in susceptible cv. Désirée in tolerant cv. Pentland Squire and in resistant cv. Santé.

Materials and methods

Seed potato plants (*Solanum tuberosum*, L.) plants were multiplied by a stem node segmentation procedure and transferred into quartz sand in a growing chamber. After 4 weeks, some plants were inoculated with PVY^{NTN}. POD were extracted and activities were measured spectrophotometrically using guaiacol described in Milavec and associates (2001). A real-time PCR assay, based on fluorogenic 5' nuclease (TaqMan®) technology, was developed to analyse the abundances of POD mRNA.

Results and discussion

Soluble, ionically- and covalently-bound POD were analysed in bottom inoculated and in upper intact leaves as well as in roots of mock (control) and virus inoculated plants 3 hours post inoculation. The changes detected in bottom inoculated leaves particular in bound POD, were cultivar specific (Fig.1).

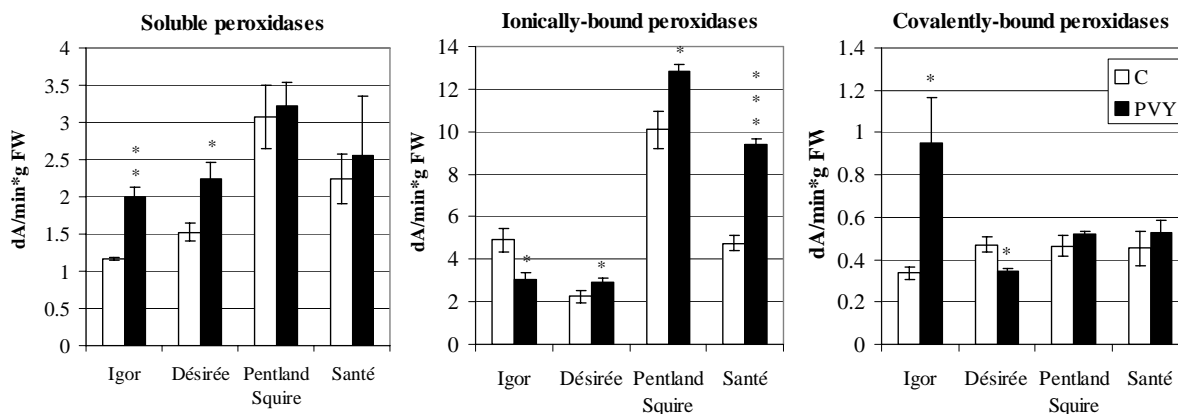


Figure 1: Peroxidase activity in bottom inoculated leaves of control (C) and infected (PVY) plants of potato cultivars Igor, Désirée, Pentland Squire and Santé 3 hours post inoculation, expressed as the change of absorption per minute per gram of fresh weight; * - statistically significant differences (* < 0,05, ** < 0,01, *** < 0,001); I - standard deviation.

In tolerant cv. Pentland Squire and resistant cv. Santé a significant increase of activity of ionically-bound POD was observed. On the contrary, in the very susceptible cv. Igor and susceptible cv. Désirée an increase of soluble POD was found. Additionally, in the very susceptible cv. Igor a decrease of activity of ionically-bound POD but an increase of activity of covalently-bound POD was detected. In upper intact leaves changes were observed only in resistant cv. Santé, where activities of soluble and covalently-bound POD increased. No changes in POD activities connected to PVY^{NTN} infection were detected in roots.

A part of new POD gene (*poxMM*) was isolated from leaves of potato cv. Igor. The nucleotide sequence of 394 bp was determined and the abundances of the POD mRNA were analysed by real time PCR. *PoxMM* was present in leaves and roots of all four potato cultivars. The abundance of the transcript was very similar in all four cultivars (Fig.2) and was more than 300 times higher in roots than in leaves. In all four cultivars no correlation between PVY^{NTN} infection and the abundance of the transcript was observed.

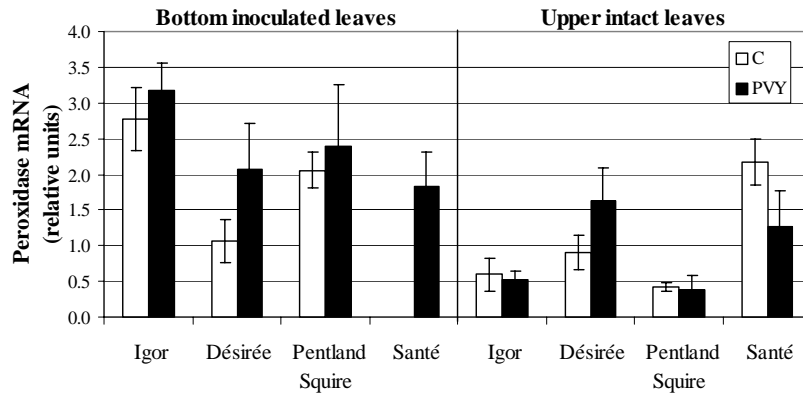


Figure 2: The abundance of peroxidase mRNA in bottom inoculated leaves control (C) and infected (PVY) plants of potato cultivars Igor, Désirée, Pentland Squire and Santé 3 hours after the inoculation, expressed in relative units; I - measurement uncertainty.

Conclusions

Changes in POD activities found in leaves 3 hours post inoculation are cultivar specific and contribute to the early response of potato cultivar to PVY^{NTN} infection. The increase of activity of ionically-bound POD might be connected to resistance and tolerance to PVY^{NTN} infection, while the increase of soluble POD is characteristic of susceptible cultivars response. The expression of *poxMM* gene is not connected to PVY^{NTN} infection.

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