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Artificial Elevation of Salicylic Acid Affects Thiol Contents and Symptom Development in *Cucurbita pepo* During Zucchini Yellow Mosaic Virus (ZYMV) Infection

By

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Summary

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Zucchini yellow mosaic virus (ZYMV) is regarded as a major pathogen of cucurbits in most regions of the world where these crops are cultivated. This paper focuses on induction of systemic acquired resistance (SAR) in ZYMV infected Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* GREB.) plants by applying different concentrations of salicylic acid (SA) to pumpkin seedlings. All SA treatments increased artificially SA and thiol contents in seedlings 48 hours after the treatment. SA treated seedlings and untreated controls were mechanically inoculated with ZYMV two weeks later. Virus inoculated plants without treatment showed severe mosaic symptoms three weeks post inoculation which were preceded by a decline in total SA levels and greater accumulation of free SA in older infected leaves. In younger infected leaves a noticeable increase of total SA was determined, whereas free SA contents decreased below the control levels. Furthermore, viral infection caused a reduction of cysteine and total glutathione levels in both older and younger leaves three weeks after ZYMV infection. SA treatment in combination with ZYMV stimulated the accumulation of both SA and thiols. Total SA contents were enhanced

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by more than 120 % after 1 mM and 3 mM SA treatment in older infected leaves with respect to infected controls. Cysteine was enhanced by 50 % in younger 1 mM SA treated leaves and 100 % in 3 mM SA treated leaves three weeks after ZYMV infection. Total glutathione levels also increased upon 0.5 mM SA treatment, but to a lesser extent than cysteine. Evaluation of symptom development revealed that SA treatment induced a delayed symptom development, a decrease in symptom severity or a complete absence of the ZYMV induced symptoms on the leaves. Three weeks after ZYMV inoculation almost 70 % of 3 mM SA treated plants did not show any signs of symptoms on the leaves, whereas all untreated and ZYMV infected plants developed yellow leaves, mosaic and green blisters. SA treatments with virus inoculation clearly showed that increased levels of SA accompanied by elevated thiol contents and provided an enhanced tolerance against ZYMV infection.

Zusammenfassung

URBANEK KRAJNC A., ZECHMANN B., STABENTHEINER E. & MÜLLER M. 2008. Artificial elevation of salicylic acid affects thiol contents and symptom development in *Cucurbita pepo* during Zucchini yellow mosaic virus (ZYMV) infection. [Die Behandlung mit Salicylsäure führt zu Veränderungen im Thiolgehalt und in der Symptomentwicklung von *Cucurbita pepo* während der Infektion mit Zuchinigelbmosaikvirus (ZGMV)]. – *Phyton* (Horn, Austria) 48 (1): 13–35.

Das Zuchinigelbmosaikvirus (ZGMV) zählt weltweit zu einem der gefährlichsten Phytopathogene an Kürbisgewächsen. Diese Arbeit beschäftigt sich mit der Induktion von systemisch erworbener Resistenz (SAR) in ZGMV-infizierten Pflanzen des steirischen Ölkürbis (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* GREB.), die mit unterschiedlichen Konzentrationen von Salicylsäure (SA) vorbehandelt wurden. Alle SA Behandlungen erhöhten nach 48 Stunden die SA Konzentration, sowie die Thiolgehalte in den Pflanzen. Zwei Wochen später wurde eine künstliche Virusinfektion der Kontrollen und der vorbehandelten Pflanzen durchgeführt. Virusinfizierte Pflanzen ohne Salicylsäurevorbehandlung zeigten drei Wochen nach der Inokulation schwere Mosaiksymptome, was mit einer Abnahme im Gesamt SA Gehalt und einer Erhöhung der freien SA in den älteren Blättern einherging. In jüngeren infizierten Blättern konnte eine Zunahme der Gesamt SA beobachtet werden, während die freie SA deutlich unter die Gehalte der Kontrollen zurückfiel. Weiters bewirkte die Virusinfektion drei Wochen später eine Reduktion des Cysteins und des Gesamtglutathions in älteren und jüngeren Blättern. Die SA Behandlung kombiniert mit der Virusinfektion stimulierte hingegen die Akkumulation von SA und Thiolen. Die Gesamt SA wurde nach 1 mM bzw. 3 mM SA Vorbehandlung um mehr als 120 % in älteren Blättern verglichen mit den infizierten Kontrollen erhöht. Cystein verzeichnete drei Wochen nach der Virusinokulation in jüngeren Blättern nach Vorbehandlung mit 1 mM SA einen Anstieg um 50 % und nach 3 mM Behandlung um 100 %. Auch Glutathiongehalte erhöhten sich nach 0.5 mM SA Behandlung. Die Evaluierung der Symptome an den Blättern bestätigte, dass SA Vorbehandlungen zu einer verzögerten Symptomentwicklung führten, wie auch zu einer Erniedrigung der Symptomstärke oder aber auch zu einem gänzlichen Ausbleiben der Symptome. Drei Wochen nach der ZGMV-Inokulation wiesen fast 70 % der mit 3mM SA vorbehandelten Pflanzen keine Symptome auf den Blättern auf, während zum gleichen Zeitpunkt die SA unbehandelten, infizierten Pflanzen gelbe Blätter, Mosaiksymp-

tome und dunkelgrüne Bläschen zeigten. SA Behandlung und Virusinfektion bewirkten eindeutig erhöhte SA Gehalte, die mit erhöhten Thiolgehalten einhergehen, was sich in einer erhöhten Toleranz der Pflanzen gegen das ZGMV niederschlug.

Introduction

Among viruses that infect cucurbit crops, Zucchini yellow mosaic virus (ZYMV) is one of the most influential viruses worldwide causing a severe economical loss in pumpkin production every year. The macroscopic visible symptoms on ZYMV infected plants appear on the leaves mostly within a week after the infection and are characterized by yellowing, blistering and mosaic symptoms. ZYMV infected plants also exhibit stunting and upright growth forms. The flowering period of the infected plants is affected by a reduction of female flowers, which may also fall off without pollination. A diversity of symptoms appears on the fruits in form of deformations and color alterations and renders them unmarketable (RADWAN & al. 2007b, URBANEK KRAJNC & al. 2007, ZECHMANN & al. 2007b).

ZYMV affects physiological processes such as antioxidant system and photosynthesis due to the pathogen-induced oxidative stress. Some enzymes are widely influenced by virus attack. For ZYMV and cucumber mosaic virus infected *Cucurbita pepo* plants, an increased activity of antioxidative enzymes, like ascorbate peroxidases, superoxide dismutases and catalase, was determined at the time of severe symptom development (RIEDLE-BAUER 2000, DE GARA & al. 2003). The marked increase in ascorbate peroxidase activity indicated, that the ascorbate glutathione cycle plays an important role in detoxification of H₂O₂. During advanced ZYMV infection decreased pigment contents, carbohydrates, aminoacids, peptides and proteins were reported (RADWAN & al. 2007b, URBANEK KRAJNC & MÜLLER 2006, URBANEK KRAJNC & al. 2007, ZECHMANN & al. 2005, 2007a,b).

Higher plants evolve complex chemical defenses against pathogens, which may deter pathogen invasion and impede harmful effects on plant health (GULLNER & KÖMIVES 2001, DE GARA & al. 2003, SHAH 2003). In plants, systemic acquired resistance (SAR) is considered a form of resistance response that occurs following an earlier localized exposure to a pathogen. It is a persistent defense strategy, which provides qualitative and quantitative changes in the chemical composition of a host plant against pathogen progression. SAR relies on the subsequent signal-transduction cascade, which involves salicylic acid (SA) as major signaling compound able to induce the expression of many defence-related genes through different pathways (MCDOWELL & DANGL 2000, KUNKEL & BROOKS 2002, SHAH 2003, LALOI & al. 2004, LOAKE & GRANT 2007, RADWAN & al. 2007b).

This paper focuses on salicylic acid-induced establishment of SAR in ZYMV infected Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var.

styriaca GREB.) plants. SA belongs to an extraordinary diverse group of plant phenolics. It accumulates locally following inoculation with pathogens inducing a special type of cell death in hypersensitive response or systemically providing SAR that immunized the entire plant against further infections (MURPHY & al. 1999, ALVAREZ 2000, OROBER & al. 2002, SHAH 2003, LOAKE & GRANT 2007). A broad range of defence mechanisms, which contribute to the appearance of the SAR include the synthesis of antioxidants and the activation of antioxidant enzymes, which appeared to quench the pathogen induced oxidative stress (FODOR & al. 1997, FRITIG & al. 1998, GULLNER & al. 1999, MURPHY & CARR 2002, OROBER & al. 2002, DE GARA & al. 2003, SMITH-BECKER & al. 2003).

One typical component of antioxidative system in plants is the low molecular weight thiol glutathione. Besides its role in scavenging reactive oxygen species it is involved in uptake, assimilation, storage and transport of sulfur and is also coupled with carbon and nitrogen metabolism in both plants and animals (NOCTOR & FOYER 1998, NOCTOR & al. 1998, 2002, FOYER & RENNENBERG 2000, FOYER & NOCTOR 2005, NOCTOR 2006). Glutathione was found to play mayor functions during virus infections (FODOR & al. 1997, GULLNER & KÖMIVES 2001, DE GARA & al. 2003, MÜLLER & al. 2005, ZECHMANN & al. 2005, 2006, 2007a,b, URBANEK KRAJNC & MÜLLER 2006, URBANEK KRANJC & al. 2007). First of all glutathione is known to protect the tissue against reactive oxygen species in a direct reaction or through the ascorbate-glutathione cycle. Furthermore, increased total glutathione (total GSH) levels were correlated with enhanced activity of glutathione S-transferase catalyzing detoxification reactions. Moreover, changes in concentration of total GSH and its redox status are known to mediate the signal effects of hydrogen peroxide and SA. Therefore, glutathione is not only an important antioxidant, but also one of the regulating chemicals determining the selectivity of responses during plant-pathogen interactions (FOYER & RENNENBERG 2000, GULLNER & KÖMIVES 2001, NOCTOR & al. 2002, DE GARA & al. 2003, MAUGHAN & FOYER 2006, NOCTOR 2006). Elevated glutathione contents have mainly been found during incompatible plant-pathogen interaction (FODOR & al. 1997, VANACKER 1998a-c, 2000), whereas in compatible plant pathogen interactions elevated glutathione levels only occurred among single, intact cells of infected Styrian oil pumpkin plants, but not in whole leaves and mosaic sections where glutathione levels remained unchanged or decreased (ZECHMANN & al. 2005, 2007a,b).

Recent analyses in which biochemical pathways have been manipulated by either feeding experiments or genetic engineering gave rise that plants with high levels of antioxidants, either constitutive or induced, have a greater resistance to virus infections (BOLTER & al. 1993, SCHNEIDER & ULLRICH 1994, GÖRLACH & al. 1996, GULLNER & al. 1999, KNÖRZER & al. 1999, ZECHMANN & al. 2005, 2007a, URBANEK KRAJNC & MÜLLER 2006,

URBANEK KRAJNC & al. 2007). In previous works, we demonstrated that treatment of Styrian oil pumpkin seedlings with L-2-oxothiazolidine carboxylic acid (OTC) subsequently elevated glutathione levels by either reducing its degradation due to the inhibition of the metabolism 5-oxo-L-proline by OTC or by serving as an intracellular delivery system for cysteine (HAUSLADEN & KUNERT 1990, FARAGO & BRUNOLD 1994). In the present investigation, the exact role of the host chemistry in defense against ZYMV was proved by manipulating signal and antioxidative pathways, as well as other physiological and chemical characteristics of Styrian oil pumpkin by exogenous application of SA. Till now exogenous SA was applied to different plant species in order to study how do plants with artificial elevated SA levels control various stress conditions (PIERPOINT 1994, FODOR & al. 1997, JAMESON & CLARKE 2002, KANG & SALTVEIT 2002, KIRÁLY & al. 2002, RADWAN & al. 2007a,b, LOAKE & GRANT 2007). Treatment with SA efficiently inhibited the virus multiplication and transport within the plant and consequently reduced the number and the size of lesions in numerous plants (SCHNEIDER & ULLRICH 1994, FODOR & al. 1997, LAMB & DIXON 1997, ALVAREZ 2000, CLARKE & al. 2002, JAMESON & CLARKE 2002, SMITH-BECKER & al. 2003, RADWAN & al. 2007b). Moreover, SA treatment caused a strong defense gene expression (MURPHY & al. 1999, LOAKE & GRANT 2007, RADWAN & al. 2007b) and induction of antioxidants, including glutathione, in virus tobacco plants (MUR & al. 1996, KIRÁLY & al. 2002). However, there is no information about the induction of thiol molecules against biotic stress by the exogenous application of SA in case of compatible virus-plant interaction.

In the present investigation, exogenous application of SA to Styrian oil pumpkin gave rise to quantitative changes in SA and thiol contents and any interactions among these molecules during ZYMV infection in the leaves near the inoculation side and in distant parts of the plant. SA treatment made it possible to establish a correlation between SA and thiols and to determine vertical rates of signal movement in pumpkins during ZYMV infection. Furthermore, it was of interest to determine the minimal concentration of exogenously applied SA, which would provide an efficient increase in SA and thiol levels in Styrian oil pumpkin seedlings, without toxic effects on plant development. The long-term effects of SA treatment on both SA and thiol levels were tested in ZYMV infected Styrian oil pumpkin plants. In addition, ZYMV induced symptom development was evaluated over a period of three weeks in order to clarify whether SA treatments provide any tolerance against ZYMV induced symptoms on the leaves.

Material and Methods

Cultivation of Seedlings and Exposure to SA

Seeds of Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* GREB.) received from Saatzeit Gleisdorf (Plant Breeding Company, Gleisdorf, Austria), were germinated in clay pots (58 x 28 cm, approximately 110 seeds, planting depth 2 cm) containing humid Perlite. The cultivation was performed in climate chambers with a photoperiod of 12 hours (6 a.m.–6 p.m., PAR 400–700 $\mu\text{mol m}^{-2} \text{s}^{-1}$, measured with a quantum sensor; Skye, UK). Day and night temperatures were 22 and 18 °C, respectively; the relative humidity was 70 %.

Two-week-old seedlings were transferred for 48 h to nylon-mesh-covered plastic dishes (500 mL) containing salicylic acid (SA) aqueous solutions with concentrations of 0.5 mM, 1.0 mM and 3.0 mM. Exposure was confined to the roots, which immersed into the solution. A minimum of 24 seedlings was used for every treatment experiment. Controls consisted in seedlings that were treated with water. The treatment experiments were conducted in growth chambers (growth conditions see above) and the SA solutions were exchanged after 24 hours.

During the treatment no toxic effects on growth and development of seedlings were observed, when seedlings were treated with 0.5 mM and 1 mM SA. In contrast, treatment with 3 mM SA emerged as lethal dose for one third of the seedlings, which wither in hypocotyl section within 12 hours.

24 seedlings of each treatment were replanted in 10 cm pots containing soil [Klasmann Substrat] at the depth of cotyledons and cultivated in two separated climate chambers under the conditions described above. Seedlings were periodically watered and after 14 days fertilized weekly.

Infection of Seedlings with ZYMV

Ten days after SA treatment, one plant group was mechanically infected with the sap of ZYMV infected plant material. For inoculation, 1 g of ZYMV infected plant material (strain id.: DSMZ PV-0466; obtained from DSMZ-Plant Virus Collection, Braunschweig, Germany) was homogenized in 1 mL of phosphate buffered saline (0.1 M PBS, pH 7.2) by adding some Celite. The inoculum was spread out on the cotyledons and the first leaf of each plant by gentle ripping the leaves with a mortar. The infection was repeated after two days. Mock inoculation was performed on control plants by rubbing the buffer with Celite onto the cotyledons and the first leaves, but without the inoculum. In the separate climate chamber the other plant group remained uninfected and was used as control group.

Symptom Characterization

To investigate the impact of SA treatment on the development of ZYMV induced symptoms, the plant group infected with ZYMV was closely monitored for any visible signs of viral symptoms one, two and three weeks after ZYMV inoculation. Symptom development was evaluated on a tripartite scale depending on the severity of the symptoms: 0 No symptoms; * first signs of yellowing on the leaves; ** advanced yellowing on the leaves, minor stunting, and first signs of mosaic pattern on the leaves; *** green blisters, leaf-deformation, severe stunting, severe mosaic pattern on the leaves (Table 3). Symptoms of 20 ZYMV infected plants derived from 4 different ex-

periments were evaluated. The number of plants showing ZYMV disease was represented in percentage.

Sample Preparation for Biochemical Analysis

One group of seedlings (24 seedlings of each treatment experiment) was harvested 48 h after SA treatment at 7.00 a.m. For further biochemical investigations, the fifth (position above hypocotyls) and the ninth leaf (the fully developed youngest leaf) each of ZYMV infected plants and controls (ten different samples of each treatment experiment) were harvested separately three weeks post inoculation, when ZYMV particles were found in all plant parts of SA treated and untreated samples and when all untreated plants showed symptoms of ZYMV disease. Plant material was immersed in liquid nitrogen immediately after harvesting. For analysis of thiols frozen samples were lyophilized and ground in a dismembrator and the resulting powder was subjected to the analysis.

Determination of Salicylic Acid

The extraction and determination of SA (free and conjugated) was principally performed according to PASQUALINI & al. 2002. SA was extracted from leaf and root samples (1–1.5 g FW) by homogenization in mortar with 2 mL 30 % (v/v) methanol in water. The homogenate was mixed by vortex for 1 minute, sonicated for 5 minutes and centrifuged at 6000 g for 2 minutes. The supernatant was collected in 10 mL tube. The pellet was re-suspended twice with 100 % methanol and the sonication and centrifugation steps were repeated. After these three extraction steps the supernatants were pooled and 10 μ L of 0.2 M NaOH was added, in order to prevent sublimation of SA (VERBERNE & al. 2002). The methanol:water mixture was concentrated to 2.5 mL at medium drying speed using a SpeedVac concentrator. The concentrated extract was divided into two aliquots. One aliquot was centrifuged and a known amount of pure SA was added to one sample whereas no SA was added to other sample (the control sample). Both extracts were used for analysis of free SA in HPLC. To determine the β -glucosylsalicylic acid content, the other aliquot of the methanolic extract (1 mL) was re-suspended in 2 mL of 8 N HCl and 1 mL of 3.7 M NaCl and hydrolyzed for 1 hour at 90 °C. The cooled mixture was then purified through a Sep-Pak C18 column. The sample was eluted by 1 mL 100 % methanol and then analyzed with HPLC: two Knauer high-pressure liquid chromatography pumps 64, Knauer Software/Hardware package, Hitachi Fluorescence Spectrophotometer F-1300 (excitation: 315 nm wavelength; emission: 405 nm wavelength); and a cooled Marathon autosampler. Column Spherisorb S5 ODS2 25 x 4.6 mm. The samples were fractionated isocratically with 45 % (v/v) methanol in water (2 % (v/v) acetic acid); flow rate was 1 mL min⁻¹.

Determination of Low Molecular Weight Thiols

Total GSH (reduced and oxidized) was determined by adapting the protocol of KRANNER & GRILL 1993. Thiols were extracted from 60 mg of lyophilized plant material in 2 mL of 0.1 M HCl with 60 mg of polyvinylpyrrolidone added to remove phenolics. 280 μ M of this extract was incubated with 420 μ L of CHES buffer [200 mM 2-(N-cyclohexylamino) ethanesulfonic acid, pH 9.0] and 70 μ L of 5 mM dithiothreitol for 1 hour at room temperature (RT) to reduce the thiol groups. The SH groups were

labeled with monobromobimane (BmBr) by incubating with 50 μL 8 mM BmBr in the dark at room temperature (RT) for 15 minutes. Derivatization was stopped by adding a 760 μL aliquot of 0.25 % (v/v) methanesulfonic acid. For the determination of the content of oxidized thiols 30 μL of 50 mM N-ethylmaleimide and 600 μL of 200 mM CHES buffer were added to 400 μL of the thiol extract, and the mixture was incubated at RT for 15 minutes in order to block the SH groups. The excess of N-ethylmaleimide was removed by extraction with toluene, and the remaining oxidized thiols were reduced by 50 μL dithiothreitol and derivatized with BmBr as described above.

Separation and determination of the derivatized thiols were done using HPLC: two Knauer high-pressure liquid chromatography pumps 64, Knauer Software/Hardware package, Shimadzu fluorescence monitor RF-535 (excitation: 380 nm wavelength; emission: 480 nm wavelength), and a cooled Marathon autosampler. Column Sphericorb S5 ODS2 25 x 4.6 mm. Solvent A: 0.25 % (v/v) acetic acid in water containing 5 % methanol, pH 3.9. Solvent B: 90 % (v/v) methanol in water; gradient: 5 % solvent B to 15 % solvent B in 20 minutes, 100 % solvent B for 6 minutes, and 5 % solvent B for another 8 minutes. Flow rate was 1 mL min⁻¹.

Statistics

The results of biochemical analyses represented medians and median deviations (M.D.) of eight replicate samples each. They were statistically evaluated with the help of the Kruskal-Wallis test, followed by post-hoc comparisons according to Conover (BORTZ & al. 2000). Significant differences were indicated by different letters (a, b), glutathione contents significantly different from each other have no letter in common. Decision rule: $P < 0.05$ was regarded as significant. Calculations were performed on the MS-STATISTICA software package (Stat-Soft, USA, 1994).

Abbreviations: BmBr, monobromobimane; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; M.D., median deviation; OTC, L-2-oxothiazolidine carboxylic acid; SA, salicylic acid; SAR, systemic acquired resistance, total GSH, total glutathione; ZYMV, Zucchini yellow mosaic virus.

Results

Biochemical Investigations on Salicylic Acid Treated Seedlings

In all SA treatments (0.5 mM, 1 mM and 3 mM SA), free and total SA contents increased significantly 48 hours after the treatment. 3 mM SA treatment increased the free and total SA contents to 47.1-fold and 112.1-fold levels, respectively (Table 1).

Seedlings also possessed a high affinity to assimilate SA into thiol compounds. In contrast to the results of SA analysis, where 3 mM SA treatment most effectively enhanced SA levels, the increase in thiol contents in response to SA treatment (Table 2) followed a saturation line upon 1 mM SA treatment. Both cysteine and total GSH contents of 1 mM SA treated cotyledons did not significantly differ from 3 mM SA treated samples. In both treatments cysteine and total GSH increased by more than

120 % in comparison to the untreated control (Table 2). The redox state of cysteine and total GSH was not affected by the treatments. γ -glutamyl-cysteine was not detected in significant amounts (data not shown).

Table 1. Effects of SA treatment on free salicylic acid (free SA) and total salicylic acid (total SA), and the differences of SA in cotyledons of two-week-old Styrian oil pumpkin seedlings calculated in comparison to the 0 mM SA 48 hours after treatment. Medians of eight replicates \pm M.D. Different letters (a, b, c) indicate significant differences ($P < 0.05$) analyzed by Kruskal-Wallis test followed by post hoc comparison according to Conover.

	C free SA	differences	C total SA	differences
0 mM SA	202.7 \pm 15.1a		836.9 \pm 201.0a	
0.5 mM SA	708.7 \pm 48.1b	3.5-fold	4280.7 \pm 535.2b	5.1-fold
1 mM SA	3363.4 \pm 405.5c	16.6-fold	12833.8 \pm 1650.8c	15.3-fold
3 mM SA	9546.1 \pm 551.3d	47.1-fold	93848.7 \pm 238.8d	112.1-fold

C – [ng g⁻¹ FW]

Table 2. Effects of SA treatment on total glutathione (total GSH), oxidized glutathione (GSSG), cysteine (cys) and cystine in cotyledons of two-week-old Styrian oil pumpkin seedlings 48 hours after treatment. Sampling was done at 7 a.m. Medians of eight replicates \pm M.D. Different letters (a, b, c) indicate significant differences ($P < 0.05$) analyzed by Kruskal-Wallis test followed by post hoc comparison according to Conover.

	C total GSH	% ¹	% GSSG	C cys	% ¹	% cystine
0 mM SA	3869.5 \pm 419.6a		31.3 \pm 8.3	550.9 \pm 254.5a		9.2 \pm 3.1
0.5 mM SA	5852.2 \pm 492.8b	51.2	31.6 \pm 17.9	1079.5 \pm 249.7ab	95.7	15.2 \pm 4.1
1 mM SA	9159.7 \pm 284.8c	136.7	31.2 \pm 5.3	1213.3 \pm 268.4b	120.1	7.5 \pm 1.1
3 mM SA	8994.6 \pm 656.6c	132.4	19.7 \pm 3.1	1344.3 \pm 358.7b	144.0	7.4 \pm 3.8

%¹ – [C (n mM SA) – C (0 mM SA)] * 100 / C (0 mM SA)

C – [nmol g⁻¹ DW]

Symptom Characterization SA pre-treated Seedlings after ZYMV Infection

Three weeks after infection with ZYMV symptoms were observed on 100 % of untreated Styrian oil pumpkin seedlings. The leaves showed yellowing, green blisters, and mosaic symptoms. Subsequently, the leaves are further reduced in size, deformed and occur often with serrated edges. Most of the infected plants exhibited stunted growth forms (Table 3).

In general, SA treatment induced delayed symptom development in comparison with others infected but untreated plants. Also, SA reduced the percentage of infection and the severity of symptoms which were examined three weeks after inoculation. Moreover, a trend towards complete

absence of ZYMV induced symptoms was observed, when plants were subjected to higher concentration of SA (Table 3). 18.8 % of 0.5 mM SA pretreated plants were characterized as healthy. However, 50 % of 0.5 mM SA treated plants showed advanced yellowing and mosaic symptoms on the leaves (disease severity ** and *** according to Table 3). 37.5 % of 1 mM SA treated seedlings did not show any symptoms. Light yellowing of the leaves was observed on 43.75 % of the plants (*, Table 3), but none of 1 mM SA treated plants showed severe mosaic symptoms. 3 mM SA treatment was the most effective one in reduction of disease severity. 68.8 % of 3 mM SA treated plants were characterized by a complete absence of ZYMV-induced symptoms on the leaves and were highly tolerant against ZYMV.

Table 3. Symptom characterization of ZYMV infected controls and SA treated Styrian oil pumpkin plants. Values represent the amount of plants three weeks after ZYMV-inoculation showing symptoms of ZYMV-disease in absolute numbers and in %.

ZYMV	Seed-derived Styrian oil pumpkin plants			
Symptoms ¹	Controls	0.5 mM SA + V	1 mM SA + V	3 mM SA + V
0	0/16 (0)	3/16 (18.75)	6/16 (37.5)	11/16 (68.75)
*	8/16 (50)	5/16 (31.25)	7/16 (43.75)	1/16 (6.25)
**	5/16 (31.25)	4/16 (25)	3/16 (18.75)	3/16 (18.75)
***	3/16 (18.75)	4/16 (25)	0/16 (0)	1/16 (6.25)

¹ Symptom-development was evaluated on a tripartite scale depending on the severity of the symptoms: 0 – no symptoms; * – first signs of yellowing on the leaves; ** – advanced yellowing on the leaves, minor stunting, first signs of mosaic patterns on the leaves; *** – green blisters, leaf deformation, severe stunting, severe mosaic patterns on the leaves.

Biochemical Investigations on Salicylic Acid Treated Seedlings three Weeks after ZYMV Infection

The effects of SA treatment, ZYMV infection and combination of both on free and total SA were determined in older and younger leaves five weeks after SA treatment and three weeks post inoculation. The corresponding leaves of uninfected plants treated with graduated concentrations of SA were used as calculation basis. All treatments without virus inoculation caused a significant increase in free SA, except of 3 mM SA treatment where free SA levels in younger leaves dropped below the controls. Total SA contents of 1 mM and 3 mM SA treated samples were enhanced to almost the same level (for 30 %) in both older and younger leaves of uninfected plants (Table 4).

ZYMV infection without SA treatment significantly affected SA metabolism. In older virus infected leaves (0 mM SA + V) the amount of free SA was significantly increased up to 65.8 %, whereas total SA decreased by 28 % in comparison to older control leaves. In younger infected leaves free

SA contents decreased below the control (-31.4 %), whereas total SA levels accumulated by 50.5 % over those of controls.

0.5 mM (SA + V) treatment significantly affected the concentrations of free SA in younger leaves, which were increased by 75.9 % in comparison to untreated ZYMV infected samples. In younger leaves a 40.8 % decline of free SA levels was measured. Total SA contents were not significantly affected by 0.5 mM (SA + V) treatment.

Table 4. Effects of SA treatment, ZYMV infection and their combination on free salicylic acid (free SA) and total salicylic acid (total SA) in older and younger leaves of Styrian oil pumpkin plants. Medians of eight replicates \pm M.D. Different letters (a, b) indicate significant differences ($P < 0.05$) within each treatment analyzed by Kruskal-Wallis test followed by post hoc comparison according to Conover; n.d. = not determined.

	C free SA	% ¹	% ²	C total SA	% ¹	% ²
0 mM SA/O	237.5 \pm 56.1a			1101.6 \pm 151.5a		
0 mM SA + V/O	393.8 \pm 99.6b	65.8		793.3 \pm 157.9b	-28.0	
0 mM SA /Y	624.6 \pm 43.2c			1315.4 \pm 139.4a		
0 mM SA + V /Y	428.8 \pm 130.0bc	-31.4		1979.4 \pm 181.5c	50.5	
0.5 mM SA/O	317.7 \pm 103.8a		33.7	1047.3 \pm 34.6a		-4.9
0.5 mM SA + V/O	233.0 \pm 28.1a	-26.7	-40.8	895.0 \pm 82.8b	-14.5	12.8
0.5 mM SA /Y	1205.0 \pm 21.7c		92.9	2169.7 \pm 106.9c		64.9
0.5 mM SA + V/Y	754.1 \pm 129.3b	-37.4	75.9	1848.7 \pm 335.5c	-14.8	-6.6
1 mM SA/O	857.4 \pm 184.3a		260.9	1404.8 \pm 69.1a		27.5
1 mM SA + V/O	358.4 \pm 51.9b	-58.2	-9.0	1744.9 \pm 279.0ab	24.2	120.0
1 mM SA/Y	957.6 \pm 189.0a		53.3	1691.7 \pm 120.4a		28.6
1 mM SA + V/Y	n.d.			1989.8 \pm 72.4b	17.6	0.5
3 mM SA/O	653.4 \pm 118.1a		175.1	1441.9 \pm 347.0a		30.9
3 mM SA + V/O	206.8 \pm 66.2b	-68.4	-52.2	1835.3 \pm 753.7a	27.3	131.4
3 mM SA/Y	255.4 \pm 73.1b		-59.1	1780.2 \pm 226.1a		35.3
3 mM SA + V/Y	617.5 \pm 253.3a	141.7	44.0	1937.2 \pm 316.2a	8.8	-2.1

C [ng g⁻¹ FW]

%¹ - $[C(n \text{ mM SA} + V) - C(n \text{ mM SA})] * 100 / C(n \text{ mM SA})$

%² - $[C(n \text{ mM SA}) - C(0 \text{ mM SA})] * 100 / C(0 \text{ mM SA})$,

$[C(n \text{ mM SA} + V) - C(0 \text{ mM SA} + V)] * 100 / C(0 \text{ mM SA} + V)$

O - older (lower) leaf

Y - younger (upper) leaf

V - virus infected leaf

In 1 mM SA treated ZYMV inoculated plants (1 mM SA + V) free SA contents did not change significantly in older leaves when compared to untreated infected leaves (0 mM SA + V). Total SA contents increased for approx. 20 % in both older and younger leaves with respect to 1 mM SA

treated plants without infection. Compared to untreated virus infected older leaves (0 mM SA + V) a 120.0 % elevation of total SA was measured in older leaves of 1 mM (SA + V) plants, whereas in younger leaves no 1 mM (SA + V) induced alterations of total SA could be observed.

In older 3 mM (SA + V) treated leaves free SA decreased by 42.3 %, whereas the total SA contents increased by 131.4 % when compared to untreated virus infected older leaves (0 mM SA + V). In younger 3 mM (SA + V) leaves free SA increased for 44.0 %, whereas total SA remained at the control level (0 mM SA + V treatment). From the data shown in Table 4 we can conclude that SA treatment dependent increase of total SA contents was most obvious in older leaves. In 1 mM and 3 mM (SA + V) infected older leaves total SA was increased by 120 % and 130 % in comparison to infected control (0 mM SA + V). Similarly as in preliminary study, gradual SA treatments in combination with virus inoculation resulted in the SA saturation, since total SA reached the same level in 1 mM and 3 mM (SA + V) treated older leaves. In upper leaves SA + V treated leaves total SA increased by 50 % over untreated younger leaves without infection, but did not accumulate to a higher extent in response to gradual SA treatment and ZYMV inoculation (Table 4).

The effects of SA treatment, ZYMV infection and the combination of both on concentrations of low molecular weight thiols in older and younger leaves are shown in Table 5. Five weeks after SA treatment no significant SA induced changes in cysteine contents were determined in older leaves of uninfected plants, whereas in younger leaves cysteine remained increased for almost 40 % and 20 % five weeks after 1 mM SA and 3 mM SA treatment. Moreover, without following inoculation a significant increase in total GSH was determined in both leaf samples of all SA treatment experiments even five weeks later. Furthermore, a gradual SA concentration dependent increase in the percentage of GSSG was noticed in older leaves. Younger leaves, which were characterized by higher GSSG contents in comparison to older leaves, showed an almost linear decline in percentage of GSSG with increased concentration of SA treatment. γ -glutamyl-cysteine was not detected in significant amount in older leaves, in younger leaves it was not significantly affected by the treatment (data not shown).

The accumulation of thiols in both older and younger leaves was much smaller, when untreated plants were infected with ZYMV. Cysteine concentrations decreased in older and younger infected leaves by more than 30 %. Total GSH dropped for 20 % (* minor symptoms) to 40 % (** severe symptoms) in both older and younger leaves of untreated plants. Samples with different values of disease severity explain the high median deviation of these results. The percentage of GSSG was similar to those of controls in both older and younger leaves.

It was noticed that all virus infected samples concerning SA treatments (SA + V) showed noticeable increased amounts of cysteine. The results are most obvious, when they are compared to infected samples without treatment (0 mM SA + V). In 1 mM (SA + V) treated younger leaves cysteine contents increased by 47.2 %, whereas 3 mM (SA + V) treatment caused 100 % elevation of cysteine levels in younger infected leaves.

In general, total GSH contents increased upon 1 mM SA + V treatment when compared to infected untreated samples (0 mM SA + V), but they remained below the total GSH level of corresponding SA treated samples without following infection. Upon 1 mM SA + V treatment a saturation of total GSH was measured, since the levels of total GSH in 1 mM and 3 mM (SA + V) treated material did not significantly differ from each other

Table 5. Effects of SA treatment, ZYMV infection and their combination on total glutathione (total GSH), percentage oxidized glutathione (GSSG) and cysteine (cys) in older and younger leaves of Styrian oil pumpkin plants. Medians of eight replicates \pm M.D. Different letters (a, b, c) indicate significant differences ($P < 0.05$) within each treatment analyzed by Kruskal-Wallis test followed by post hoc comparison according to Conover.

	Ctotal GSH	% ¹	% ²	% GSSG	Ccys	% ¹	% ²
0 mM SA/O	3407.0 \pm 175.7a			11.0 \pm 4.9	148.7 \pm 49.8a		
0 mM SA + V/O	2571.6 \pm 986.3a	-24.5		20.4 \pm 9.8	97.7 \pm 24.2a	-34.3	
0 mM SA /Y	4129.0 \pm 88.8b			42.3 \pm 10.4	126.9 \pm 28.8a		
0 mM SA + V /Y	3415.8 \pm 344.7a	-17.3		49.4 \pm 11.7	79.2 \pm 42.7a	-37.6	
0.5 mM SA/O	4424.7 \pm 263.5a		30.0	21.7 \pm 10.3	161.2 \pm 20.9a		8.4
0.5 mM SA + V/O	2453.8 \pm 646.9b	-44.6	-4.6	52.7 \pm 11.1	126.4 \pm 37.6a	-21.6	29.4
0.5 mM SA /Y	5145.3 \pm 495.1a		24.6	23.1 \pm 5.6	139.8 \pm 30.2a		10.2
0.5 mM SA + V/Y	3033.7 \pm 473.1b	-41.0	-11.2	53.7 \pm 10.2	90.9 \pm 29.0a	-35.0	14.8
1 mM SA/O	4142.0 \pm 192.9ab		21.6	36.1 \pm 12.8	140.3 \pm 26.8a		-5.6
1 mM SA + V/O	3556.8 \pm 356.7b	-14.1	38.3	54.2 \pm 11.7	120.2 \pm 24.3a	-14.3	23.0
1 mM SA/Y	5243.1 \pm 252.6a		27.0	25.0 \pm 13.2	176.3 \pm 58.9a		38.9
1 mM SA + V/Y	4177.2 \pm 565.5ab	-20.3	22.3	24.4 \pm 10.5	116.6 \pm 30.4a	-33.9	47.2
3 mM SA/O	5621.1 \pm 192.7a		65.0	44.6 \pm 13.3	183.7 \pm 45.2a		23.5
3 mM SA+ V/O	3390.6 \pm 512.8b	-39.7	31.8	34.1 \pm 8.6	111.6 \pm 8.6b	-39.3	14.2
3 mM SA/Y	5545.7 \pm 155.5a		34.3	16.9 \pm 6.2	152.3 \pm 17.2a		20.0
3 mM SA + V/Y	4302.2 \pm 790.3ab	-22.4	26.0	39.7 \pm 11.3	158.4 \pm 43.0a	4.0	100.0

C [nmol g⁻¹ DW]

%¹ - [C (n mM SA + V) - C (n mM SA)] * 100 / C (n mM SA)

%² - [C (n mM SA) - C (0 mM SA)] * 100 / C (0 mM SA),

[C (n mM SA + V) - C (0 mM SA + V)] * 100 / C (0 mM SA + V)

O - older (lower) leaf

Y - younger (upper) leaf

V - virus infected leaf

(Table 5). Similar effect was observed in preliminary study of total GSH (Table 2) and in case of SA within (SA + V) samples. In both 1 mM SA + V and 3 mM SA + V treated samples total GSH increased for more than 30 % in older leaves and 20 % in younger leaves when the contents were compared to virus infected control samples (0 mM SA + V).

Discussion

In the present investigation, SA was applied to Styrian oil pumpkin seedlings in order to sufficiently increase plant defense mechanisms against ZYMV and suppress the ZYMV induced symptoms on leaves. The results demonstrated that the artificial elevation of SA is accompanied by increased thiol contents. These SA dependent changes in the metabolic processes resulted in reduction of disease severity when plants were infected with ZYMV. SA was previously implicated in protecting *Cucurbita pepo* from infestation by ZYMV (RADWAN & al. 2007a,b), but the spatial and temporal gradation of SA and related thiol molecules has not been proved yet.

In the present study, SA was exogenously applied for 48 hours, due to the previous experiments done on cucumber, which described that the local and systemic accumulation of free SA was maximal 48 hours after the application with SA (OROBER & al. 2002). In all SA treatments (0.5 mM, 1 mM and 3 mM SA), total SA contents increased significantly 48 hours after the treatment and the percentage of free SA did not differ from control levels. Previous studies suggest the free SA as the active form is transported across plasma membranes. It is responsible for SA function in cells, which has been demonstrated to elevate active oxygen species under stress conditions (CHEN & al. 2001). The conversion of SA to glycosides may enable the phytotoxic accumulation of free SA during SA treatment (JAMESON & CLARKE 2002).

The treatment of seedlings with SA had positive effects on glutathione levels, reflecting that plants may possess a high affinity to assimilate SA into thiol compounds. Total GSH levels were significantly elevated by more than 130 % in cotyledons upon 1 mM SA treatment. This results are in agreement with previous studies suggested a close relationship between SA and glutathione pathways (FODOR & al. 1997, CHEN & al. 2001, FREEMANN & al. 2005). It was reported that tobacco leaves injected with 0.8 mM SA respond with a rapid elevation of total GSH (70 %) at the site of application (FODOR & al. 1997). On the other side reduced glutathione (0.75 mM), also caused a significant elevation of intracellular SA in tobacco cell suspension culture (CHEN & al. 2001). Furthermore, FREEMANN & al. 2005 reported that SA hyperaccumulation in *npr1-1 Thlaspi* mutants was associated with glutathione accumulation. The authors present evidence that the glutathione-mediated Ni tolerance mechanism is signaled

by the constitutively elevated levels of SA. They proposed that constitutively elevated SA acts to posttranslationally up-regulate serin acetyltransferase activity, causing constitutively elevated glutathione. Serin acetyltransferase catalyzes the acetylation of L-Serin to produce O-acetyl-L-serine, which acts as both a key positive regulator of sulfur assimilation and forms the carbon skeleton for cysteine biosynthesis (FREEMANN & al. 2004, 2005).

Interestingly, in our study the accumulation of total GSH was identical in both 1 mM and 3 mM SA treated cotyledons. It could be expected that in 3 mM SA treated plants glutathione synthesis was saturated by feed-back regulation, due to the complex regulatory mechanisms of thiols (NOCTOR & FOYER 1998, NOCTOR & al. 1998, 2002, FOYER & RENNINGER 2000, FREEMANN & al. 2004, NOCTOR 2006). However, the saturation upon 1 mM SA treatment was not observed in case of cysteine, a direct precursor of glutathione, which increased linearly with higher concentration of SA. It seems that the excess of cysteine was further transported, degraded or incorporated into other synthetic pathways. The redox state of glutathione and cysteine in cotyledons was not affected by the treatments, reflecting that SA at concentration of 1 mM and 3 mM did not cause oxidative stress and thus damaging effects on the cellular level in leaves. Furthermore, treatment with 3 mM SA for 48 h did not cause any long-term negative effects such as visible damage on the plant or on the ultrastructure (data not shown). This is the most important requirement in manipulating biochemical pathways in realistic experimental settings. Previous SA treatment experiments made by other authors showed that the toxic dose of SA depends on plant species. In mustard seedlings (DAT & al. 1998) and tobacco plants (FODOR & al. 1997) treated with increased concentration of SA, glutathione pool of the leaves became more oxidized. On tobacco leaves these phytotoxic effects were visible at concentrations higher than 1.2 mM (FODOR & al. 1997, KIRÁLY & al. 2002), growth and transpiration rate of bean (*Vicia faba*) seedlings was negatively affected at SA concentrations higher than 3.5 mM (MANTHE & al. 1992), whereas for leaf discs of *Gaultheria procumbens* the lethal dose was at 10 mM (PIERPOINT 1994). Therefore, SA treatment in the concentration used in the present study is well suited for substantially elevating SA and thiol levels in cucurbit plants. This can be traced back to the fact that to the conversion of SA to glycosides may block the phytotoxic accumulation of free SA during SA treatment (CHEN & al. 2001, JAMESON & CLARKE 2002).

The SA treatment in combination with ZYMV resulted in a surprising long-term effect on symptom development. Three weeks after ZYMV inoculation, when all untreated plants showed strong symptoms of ZYMV disease, only 30 % of SA-treated plants showed symptoms. The present results are in agreement with the opinion that SA could indirectly influ-

ence the infectivity of the virus, especially at high concentrations (FODOR & al. 1997, JAMESON & CLARKE 2002, KIRÁLY & al. 2002, RADWAN & al. 2007a,b). It has been previously reported on tobacco mosaic virus infected tobacco plants, that SA treatment at levels of 0.8 mM contributed to the suppression of necrotic symptom development (FODOR & al. 1997, KIRÁLY & al. 2002). SA dependent reduction in disease severity was also reported by RADWAN & al. 2007a in case of *C. pepo* cv Eskandarani, although SA was sprayed onto pumpkin leaves at lower concentrations (10–100 μ M SA). Suppression of the ZYMV induced symptoms on the leaves indicated that SA might play a certain role by alleviating photosynthesis inhibition caused by ZYMV infection (RADWAN & al. 2007a,b). RADWAN & al. 2007a suggested that the role of SA in inducing plant defense mechanisms against ZYMV infection might occur through the SA-antioxidant system. Such interference might occur through activation of some antioxidant enzymes. Our study confirms this presumption since the increased amount of SA in Styrian oil pumpkin plants were accompanied by elevation of thiol content during ZYMV infection. As described in our preliminary experiment above, the SA dependent accumulation of glutathione is attributed to posttranslationally up-regulation of serin acetyltransferase activity, which indirect increases cysteine and glutathione levels (FREEMAN & al. 2004). During ZYMV infection this SA-thiol interaction could be directly responsible for delayed symptom development and the reduction in the number and the size of the symptoms at all SA concentrations in long term. In future further genetic, genomic and biochemical approaches should be implicated in the dissection of the SA-dependent defense responses (JAMESON & CLARKE 2002, SHAH 2003, FREEMAN & al. 2004, 2005).

Biochemical analysis of SA three weeks after ZYMV inoculation revealed that total SA levels were significantly decreased in untreated plants (0 mM SA + V) in lower leaves. Decreased contents of total SA in older leaves could reflect a rapid conversion and translocation of SA to the site of infection. The increased conversion rate in lower leaves could be confirmed with higher accumulation of free SA, which acts as the active form of SA and is directly involved in triggering oxidative stress and in induction of SAR (SHULAEV & al. 1995, LAMB & DIXON 1997, RIEDLE-BAUER 2000, CHEN & al. 2001, SHAH 2003, LOAKE & GRANT 2007). It is generally accepted, that free SA accumulated at higher level at and near the site of infection (ORBER & al. 2002).

Contrary results were obtained for upper leaves, which accumulated higher amounts of total SA in response to ZYMV, whereas free SA dropped below the control level. It could be expected that younger leaves are mainly involved in synthesis and accumulation of SA, which could be converted and mobilized in case of detoxification reactions (e.g. SHULAEV & al. 1995, CHEN & al. 2001, SHAH 2003, LOAKE & GRANT 2007). Our results

are consistent with SA kinetics reported for TMV infected tobacco leaves, where no significant changes in concentrations of total SA were determined in the middle leaves, whereas markedly increased contents of total SA were found in the eighth leaf (SHULAEV & al. 1995).

The biochemical analysis of thiols revealed that the accumulation of total GSH and cysteine was much smaller in both older and younger leaves, when untreated plants were infected with ZYMV (0 mM SA + V), probably due to diminishing capacity of the leaf tissue to resist oxidative stress (ZECHMANN & al. 2005, 2007a,b). The decreased ability of the plant to quench oxidative stress in older leaves might be responsible for development of discoloration of the leaves, malfunctions and growth abnormalities of systemically ZYMV infected plants, which resulted in ZYMV induced senescence and cell death. This was previously confirmed by RADWAN & al. 2007b, which showed that ZYMV infection decreased pigment, protein and carbohydrate levels. Decreased concentrations of thiols in ZYMV infected plants corresponded with investigations on different plants using highly virulent fungal species, which also caused marked reductions in total GSH contents (GULLNER & KÖMIVES 2001). They are further consistent with numerous medical reports since viruses like human immunodeficiency virus (HIV) and herpes simplex progressively deplete total GSH content in different cell tissues of humans (RODRIGUEZ & al. 1998, JAHOR & al. 1999, CHOI & al. 2000). As glutathione is synthesized out of its constituents cysteine, glycine and glutamate, we can suppose that the decline of cysteine observed during ZYMV attack could be responsible for lower total GSH contents during the course of infection. A depletion of glutathione, cysteine and other precursors of glutathione synthesis (glutamate and glycine) in Styrian oil pumpkin plants during ZYMV was also confirmed on the subcellular level using selective antibodies. Generally, levels of cysteine were found to be strongly decreased in most of cell compartments; however the strongest decrease was found in plastids of older and younger leaves (ZECHMANN & al. 2007a), reflecting chlorophyll bleaching and discoloration of the leaves in the long term. Considering the above described results, the availability of glutathione precursors in leaves is essential for glutathione synthesis, especially in stress situations, and affects the ability of the plant to fight oxidative stress (NOCTOR & FOYER 1998, NOCTOR & al. 1998, 2002, KOPRIVA & RENNENBERG 2004, FREEMAN & al. 2004, 2005, NOCTOR 2006).

The biochemical investigations on the SA + ZYMV infected older and younger pumpkin leaves confirmed the previous studies that exogenous SA induces defense responses and is required for both localized resistance and systemic acquired resistance (LAMB & DIXON 1997, ALVAREZ 2000, SHAH 2003, FREEMAN & al. 2004, 2005, LOAKE & GRANT 2007). Our main research priority was to study the effects of SA treatment, ZYMV infection and the

combination of both on concentrations of SA and low molecular weight thiols. Five weeks after SA treatment, a trend towards increased concentrations of total SA and thiol compounds was determined among SA treated plants without infection. This increase reflects the long term effect of SA on the synthesis of SA and thiol molecules.

Infection of Styrian oil pumpkin seedlings with ZYMV in combination with SA treatment provided significant changes in endogenous SA levels similar to what was described for other pumpkin species, tobacco and cucumber (MÖLDERS & al. 1994, 1996, FODOR & al. 1997, OROBER & al. 2002, RADWAN & al. 2007a,b), indicating that these plants are likely to be responsive to systemic increases in endogenous SA. In SA treatment experiments a trend towards increased levels of total SA was determined in lower leaves of ZYMV infected plants with increased concentration of SA treatment. Contrary, in upper leaves total SA seemed not to be affected by SA treatment. These results indicate that SA + V treatment affected the translocation and synthesis of SA on behalf of lower leaves near the site of infection (e.g. SHULAEV & al. 1995).

Furthermore, treatment with graduated concentrations of SA increased the total GSH and cysteine contents in ZYMV infected plants. In 3 mM SA treated ZYMV infected leaves total GSH increased by 30 % and cysteine by 100 % over untreated ZYMV infected samples. The results showed that SA + V treatment triggers the accumulation of cysteine rather than glutathione. A high proportion of GSSG observed in SA + V leaves indicates a perturbation in the cellular redox state during the compatible interaction. It could be related to a higher activity of glutathione S-transferase and ascorbate glutathione cycle during oxidative stress caused by ZYMV, reflecting the important protective role of glutathione to quench the oxygen species within the cells (e.g. GULLNER & KÖMIVES 2001, FREEMAN & al. 2004, 2005, NOCTOR 2006). In our previous study, thiols were also artificially elevated by OTC. Similarly, 1 mM OTC treatment resulted in higher glutathione contents in older and younger Styrian oil pumpkin leaves five weeks after ZYMV infection (URBANÉK KRAJNC & al. 2007, ZECHMANN & al. 2007b). Both SA and OTC treatment experiments on Styrian oil pumpkin plants reflected that cysteine might be a limiting factor for glutathione synthesis (ZECHMANN & al. 2007a,b). As glutathione levels have been found to protect plants against pathogen attack, it is possible that elevated glutathione levels suppress symptom development by either detoxifying pathogen-induced reactive oxygen species or by activating defense genes (GULLNER & KÖMIVES 2001, DE GARA & al. 2003, FREEMAN & al. 2004, 2005, MAUGHAN & FOYER 2006, RADWAN & al. 2007a). Temporal distribution of thiol contents during SA treatment was previously studied on tobacco plants, in case of incompatible plant-pathogen interaction. They showed a rapid elevation of thiol contents at the site of

SA injection (0.8 mM SA), but ten days after application the levels were comparable to controls (FODOR & al. 1997). In contrast, our study confirms the long term effect of SA treatment on thiol accumulation over a period of five weeks.

Summing up, ZYMV infection decreased the total SA and stimulated its conversion to free SA in lower leaves, whereas in upper leaves total SA increased significantly. The thiol contents dropped in both lower and upper infected leaves. These negative effects could be reversed by exogenous application of SA. The increased levels of SA and thiols in SA treated leaves could be directly responsible for the observed suppression, reduction and delay of symptom development during compatible virus attack. The cross-talk between SA and glutathione seems to result in H₂O₂ accumulation and in the induction of other defense molecules (FODOR & al. 1997, KUNKEL & BROOKS 2002, SHAH 2003, FREEMAN & al. 2004, 2005, LALOI & al. 2004, RADWAN & al. 2007a,b). The results showed SA and thiols as important indicators for biotic stress and SAR development. Induction of these molecules could provide a potentially stable and ecologically acceptable solution for minimizing disease progression on a large variety of economically important crops.

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