

An insight into the defense mechanisms and the role of glutathione during advanced ZYMV infection in Styrian oil pumpkin

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This article reviews the current knowledge of the Zucchini yellow mosaic virus (ZYMV) specific physiological alterations within Styrian oil pumpkin plants with respect to glutathione metabolism. Reactive oxygen species (ROS), whose formation is accelerated under the virus infection, must be rapidly processed by antioxidants if oxidative damage is to be averted. Coupled with carbon, nitrogen and sulfur metabolism the reduced form of glutathione is known to play important protective roles in responses against viral pathogen infections, acting as an antioxidant, signal molecule and redox buffer. Biochemical analysis and immunocytochemical techniques were used, in order to demonstrate the alterations in the concentration and the distribution of glutathione in organs, tissues and within single cells of ZYMV infected Styrian oil pumpkin plants. The symptoms were preceded by a decline in total glutathione levels within whole leaves, whereas in single cells of dark-green leaf parts, close to the main leaf vein, increased glutathione concentrations were observed. Besides genetic engineering, possibilities to increase cellular glutathione contents in plants are the use of glutathione precursors or chemicals, which are stimulating glutathione synthesis. Experiments with L-2-oxothiazolidine-4-carboxylic acid (OTC) and salicylic acid (SA) demonstrated, that both chemicals were rapidly taken up by the plants since cotyledons, roots, leaf protoplasts and callus cells possessed a high affinity to assimilate these chemicals into thiol compounds. Furthermore, treatment with OTC and SA provided an enhanced tolerance against ZYMV induced symptoms and stimulated the synthesis, conversion and translocation of glutathione during advanced ZYMV infection in seedlings and regenerated plants. Since the characteristically thin, coated seed character of the Styrian oil pumpkin is a genetically recessive trait somatic embryogenesis discussed in this article would be important not only to obtain virus resistance by modulating the physiological properties of the plantlets, but also for the conservation of this local cultivar.

Key words: Cucurbita pepo, Styrian oil pumpkin, ZYMV, reactive oxygen species, systemic acquired resistance, glutathione, L-2-oxothiazolidine-4-carboxylic acid, salicylic acid, somatic embryogenesis, virus tolerance
Abbreviations: GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; HIV, human immunodeficiency virus; H₂O₂, hydrogen peroxide; OTC, L-2-oxothiazolidine-4-carboxylic acid; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; ZYMV, Zucchini yellow mosaic virus;

INTRODUCTION

Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) is an important cultivar in north-eastern parts of Slovenia and the neighboring regions of Austria and Hungary. The main characteristic of this local cultivar are the dark green colored thin coated seeds. They are used for the production of the pumpkin seed oil, which is not only a regional specialty, but has also gained increased medical attention and international popularity (Broadbent and MacLean 1998; Wagner 1998, 2000). The appearance of the thin coated mutant in Styria (Slovenia, Austria) in the late 19th century has

brought much relief in the production of pumpkin seed oil, because it was not more necessary for farmers to crack the thick shells from the seeds by hand (Teppner 1999, 2000). The production of Styrian oil pumpkin increased drastically in the sixties of the last century, when the pumpkin harvesting machine was applied into pumpkin industry. Another rapid increase in the pumpkin seed oil production was gained through European Union and their promotion of Styrian oil pumpkin production (Weber 1998; Liebhard et al. 2000).

In the last few years, the Styrian oil pumpkin cultivation has declined in productivity and quality. The major problem in cultivation is the outbreak of the Zucchini yellow mosaic virus (ZYMV), which causes severe economical losses annually (Weber 1998; Liebhard et al. 2000). Since it was first described in Northern Italy in 1973 by Lisa et al. (1981), it has been found throughout the temperate regions of the world infecting economically important cucurbits and inducing diversity of symptoms (Lisa and Lecoq 1984; Desbiez and Lecoq 1997).

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The macroscopic visible symptoms on ZYMV infected Styrian oil pumpkin plants appear on the leaves mostly within a week after the infection and are first of all characterized by yellowing, blistering and mosaic symptoms. Subsequently, the leaves are further reduced in size, deformed and occur often with serrated edges. 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 the form of deformations and color alterations and renders them unmarketable (Fuchs 2001; Zechmann et al. 2003). The symptom appearance is obviously regulated by more or less balanced interactions between host defense mechanisms, virus and environmental factors (Lesemann 1999).

The present paper discusses the current knowledge of physiological changes in Styrian oil pumpkin plants during the infection by ZYMV. One component of defense systems is the low molecular weight thiol glutathione, which is involved in numerous physiological functions acting as antioxidant, signal molecule and redox buffer (e.g. Foyer and Rennenberg 2000; Noctor et al. 2002; Noctor 2006). Attention is given to ZYMV affected alterations in glutathione levels within organs, tissues and single cells. The impact of L-2-oxothiazolidine-4-carboxylic acid (OTC) and salicylic acid (SA) treatment on the glutathione levels and the resistance to ZYMV infection is discussed.

General physiological changes in Styrian oil pumpkin plants caused by ZYMV

ZYMV is, like other viruses, an obligate parasite, which uses the plant cells as biochemical and molecular environment. It disturbs the biochemical balance of the host cells since host cell components have to be used for the synthesis of viral components and the formation of new infective viral particles (Lesemann 1999). Upon ZYMV infection, a multi-component defense response is initiated, whose individual molecules are activated in a spatial and temporal complex pattern. The common mechanism associated with an active defense includes the generation of reactive oxygen species (ROS), the induction of local cell death (hypersensitive response), the synthesis of pathogenesis-related proteins and the activation of systemic acquired resistance (SAR). Oxidative burst caused by ROS is one of the earliest reactions detectable after virus infection characterized by formation of hydrogen peroxide (H_2O_2), superoxide ion (O_2^-), hydroxyl radical (OH^-) and singlet oxygen (Clarke et al. 2002; Foyer and Noctor 2003). The increased levels of H_2O_2 are crucial in the primary resistance response to pathogen infection (Kiraly et al. 2002; Goldbach et al. 2003). Generation of H_2O_2 is known to be caused by SA, which binds specific to catalase resulting in a decline in its activity and increased H_2O_2 accumulation. H_2O_2 plays a dual role in the oxidative burst, acting as a signal for localized death within cells at high concentrations and as a diffusible signal for the induction of defense genes encoding pathogenesis-related proteins (Clarke et al. 2002; Foyer and Noctor 2003). The systemic expression of these genes is accompanied by onset of SAR (Clarke et al. 2002; Kiraly et al. 2002), which could be compared with the immunization in animals and humans. It gradually develops

throughout the plant and protects the plant against the systemic spread of the invading pathogen (Mölders et al. 1996; Kovač 2002). The broad range of defense mechanisms, which contribute to the appearance of SAR, provide different functions, such as the protection against excess energy absorption, alternative regeneration of the electron acceptor NADP, the scavenging of ROS, the repair of oxidatively damaged structures and regulation processes (Shirazu and Schulze-Lefert 2000; Jameson and Clarke 2002; Noctor et al. 2002). These mechanisms include the synthesis of antioxidants such as ascorbate and glutathione, and the activation of enzymes such as ascorbate and glutathione peroxidases, glutathione reductase (GR), glutathione S-transferase, catalases and superoxide dismutases (SOD) (Riedle-Bauer 2000, Foyer and Rennenberg 2000, Clarke et al. 2002). These components function as direct radical scavengers or are able to keep the antioxidants in the reduced form. For the ZYMV infected Styrian oil pumpkin plants an increased activity of ascorbate peroxidases, SOD and catalase, was determined during advanced symptom expression. The marked increase in ascorbate-peroxidase activity indicated, that the ascorbate-glutathione cycle in chloroplasts might play an important role to prevent destructive oxidative processes (Riedle-Bauer 2000). Furthermore, the infected leaves are characterized by increased respiration and decreased net photosynthetic rates. These physiological imbalances are accompanied with alterations in sugar composition, carbon assimilation and metabolism (Shalitin and Wolf 2000).

For infected tobacco leaves large quantities of ROS are known to accumulate locally around the tobacco mosaic virus initiating lipid peroxidation, protein oxidation, damage of nucleic acids and structural membranes and cell necrosis (Fodor et al. 1997; Riedle-Bauer 2000). This hypersensitive response is characteristic for incompatible virus-host interactions and restricts the virus to certain cells and organs. In contrast, a systemic spread of ZYMV is noticed in Styrian oil pumpkin (compatible virus-host interactions). Two possible reasons for virus propagation are addressed, either the development of ROS is not sufficient for tissue collapse, or that the enhanced antioxidant levels inhibit local cell death. In summary, it seems that the balance between increased oxidative burst and levels of antioxidants, influences the infection process and the development of symptoms. During systemic virus spread color deviations are among the first symptoms that can be observed. In leaves they are mostly due to chlorophyll disorders, which are often restricted to certain parts of the leaf giving rise to a mosaic pattern. Furthermore, increased lipid peroxidation, and advanced disintegration of membranes was observed in ZYMV infected Styrian oil pumpkin plants. Discolorations are often accompanied by a marked change in the metabolism of the hormones, which account for growth reductions and malfunctions often caused by hormone disturbances in infected plants (Riedle-Bauer 2000; Gullner and Kömives 2001; Jameson and Clarke 2002).

Cellular defense and protection mediated by glutathione

Glutathione is a crucial part of the antioxidative system in plants and animals (Foyer and Rennenberg 2000; Noctor et al. 2002; Noctor 2006). It is known to scavenge ROS in direct reactions (Winterbourn and Metodiewa 1999) or through the ascorbate-glutathione cycle (Figure 1), which represents an alternative enzymatic mechanism for removing H_2O_2 by successive oxidations and reductions of glutathione, ascorbate and NADPH in chloroplasts (Foyer and Halliwell 1976).

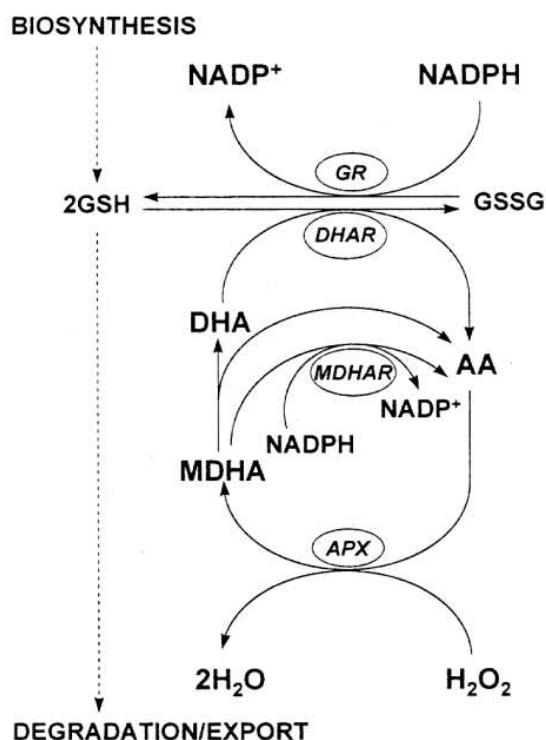


Fig. 1. The schematic representation of the ascorbate-glutathione cycle, which supposed to be the main way of removing H_2O_2 and simultaneously regenerating the electron acceptor NADP. GSH is essential for the enzyme-catalyzed regeneration of dehydroascorbate (DHA). In this reaction it will be oxidized to GSSG and regenerated using reducing power from the photosynthetic electron transport chain (NADPH). Besides the activities of enzymes the capacity of the ascorbate-glutathione cycle may depend on glutathione biosynthesis and degradation/export (according to Noctor and Foyer 1998). AA=ascorbic acid, APX=ascorbate peroxidase, DHA=dehydroascorbate, DHAR=dehydroascorbate reductase, GSSG=oxidized glutathione, GSH=reduced glutathione, GR=glutathione reductase, MDHA=monodehydroascorbate, MDHAR=monodehydroascorbate reductase.

Glutathione is mainly synthesized in the leaves and is translocated in the phloem to regulate sulfate uptake at the roots (Foyer and Rennenberg 2000; Herschbach et al. 2000; Foyer and Noctor 2001). In green plant tissues, the glutathione pool is kept to 90 % in the reduced state and is remar-

kably constant, due to the strict homeostatic control (Noctor 2006). The oxidized form of glutathione is glutathione disulphide (GSSG). Under oxidizing conditions the proportion of GSSG may be increased (Foyer and Rennenberg 2000; Tausz et al. 2000; Tausz and Grill 2000; Noctor et al. 2002). Signaling, initiated by changes in the redox state, may lead to up-regulation of glutathione synthesis and increases in the total concentration (Noctor 2006). The oxidized form of glutathione may be converted back to the reduced form (GSH) by enzyme GR [$GSSG + NADPH + H^+ \rightleftharpoons 2GSH + NADP^+$] (Foyer and Halliwell 1976).

Due to the antioxidative properties, glutathione protects proteins against the denaturation that is caused by oxidation of protein thiol groups during stress and acts therefore as a redox buffer (Noctor et al. 2002; Noctor 2006). Furthermore, glutathione stabilizes membrane structures, since membrane lipids are a prominent target for ROS reactions and lipid peroxidation forms acyl peroxides, which can be removed by glutathione (McKersie and Leshem 1994; Tausz 2001). Beside its role as a major antioxidant, glutathione synthesis is essential for utilization of intermediates from the photorespiratory cycle (Noctor and Foyer 1998).

Glutathione pool is also involved in signaling biological stress by the regulation of gene expression and protein function. High proportion of GSSG initiates signaling cascades and can regulate GR expression (Foyer and Noctor 2003; Noctor 2006). A further, more important mechanism of GSSG in defense action, involves the spontaneous oxidation of protein sulphhydryl groups to form mixed disulfides (Figure 2), a reaction termed thiolation. Reversible protein thiolation protects essential thiol groups on key proteins from irreversible inactivation during oxidative stress and also plays an important regulatory role in controlling metabolism, protein turnover, gene transcription and signal transduction pathways that initiate key stress responses (Foyer and Noctor 2001; Noctor et al. 2002).

Impacts of ZYMV infection on glutathione levels in Styrian oil pumpkin

Glutathione concentration and redox state within the ZYMV infected Styrian oil pumpkin plants are thought to be determined by glutathione synthesis, utilization and degradation, its long-distance transport, and its metabolic conversion to other compounds due to the propagation rates or degree of virus spread within the plants (Zechmann et al. 2005). Significant changes of glutathione contents were observed within organs, tissues and within different cells of Styrian oil pumpkin three weeks after ZYMV infection, when severe mosaic symptoms on leaves appeared (Urbanek Krajnc 2004; Zechmann et al. 2005). Biochemical investigations determined significantly lower levels of total glutathione in both older and younger leaves. The percentage of GSSG was not affected by the infection (Urbanek Krajnc 2004). Deficiency of glutathione within plant cells demonstrates the need of the plant for cellular protection against ROS (Foyer et al. 2001). It could be confirmed with recent investigations, where oxidative stress, caused in the presence of artificial elevated H_2O_2 levels, induced a degradation of glutathione (Lappartient and Touraine 1997; Müller et al. 1999). In Styrian oil pumpkin plants glutathione seemed to be a limiting factor for operation of the ascorbate-glutathione cycle and detoxifica-

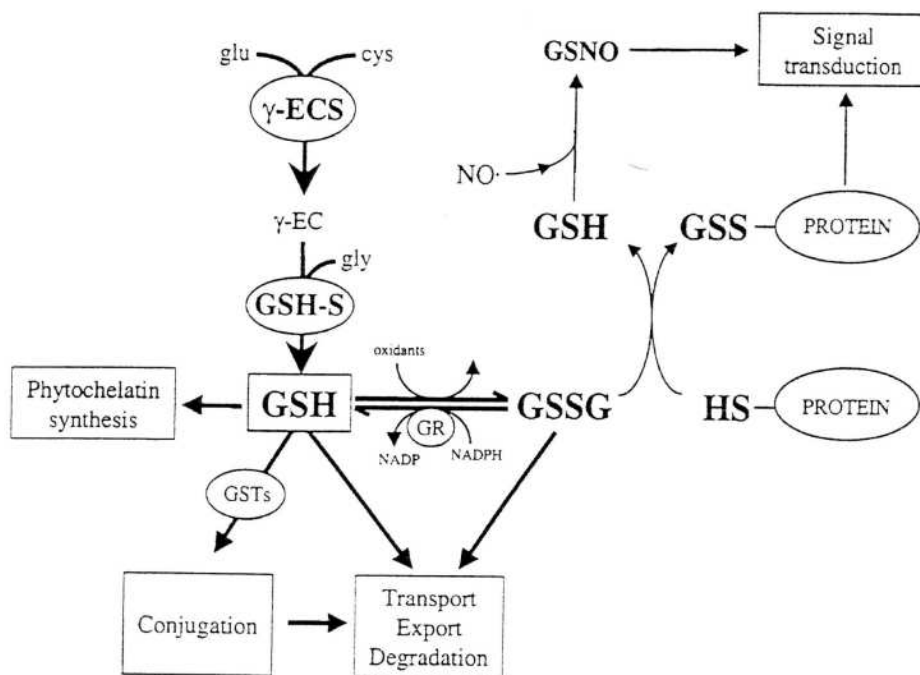


Fig. 2. Glutathione biosynthesis and some interacting processes in plant cells (according to Noctor & al. 2002). -EC = -glutamylcysteine, -ECS = -glutamylcysteine synthetase, GR = glutathione reductase, glutathione = reduced glutathione, GSSG = oxidized glutathione, GSH-S = glutathione synthetase, GST = glutathione S-transferase.

tion reactions catalyzed by glutathione S-transferase during the advanced stage of infection development. This results in a progressively diminishing capacity of the leaf tissue to resist oxidative stress and to repair damage by regenerating protein sulphhydryl groups and show up as externally visible symptoms (Figure 3) (Gönner and Schlösser 1993; Kuzniak and Sklodowska 1999).

tissue, showed increased gold-particle density in mesophyll cells of lower and upper leaves of ZYMV infected pumpkin plants (Zechmann et al. 2005). These contrary results could be explained by the hypothesis that glutathione induced resistance to ZYMV can operate at the single-cell level and that glutathione could respond on the same pathogen in different cell types with different capacity to resist oxidative stress,

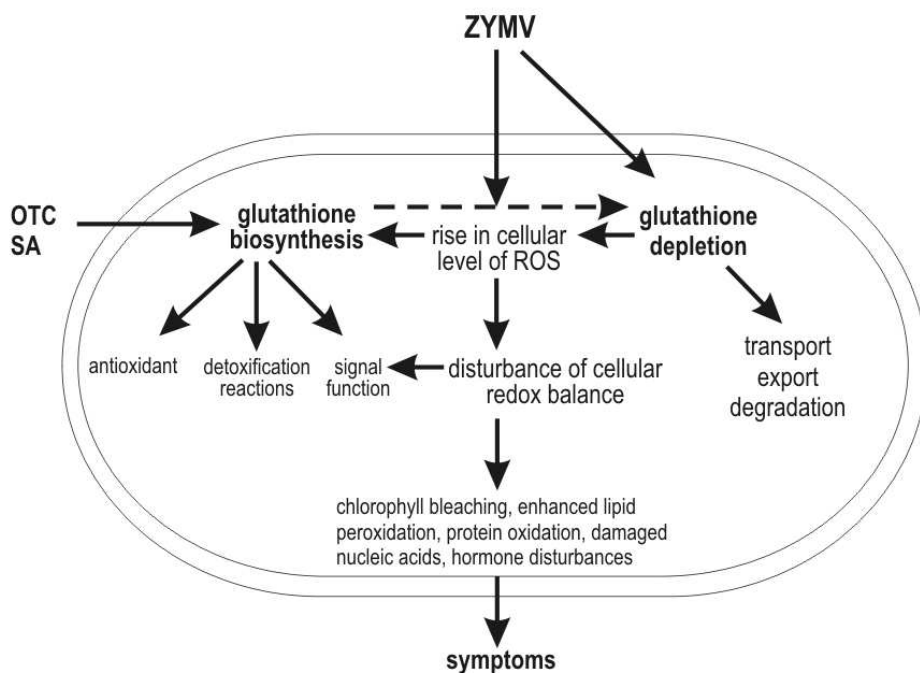


Fig. 3. Possible effects of ZYMV infection in combination with treatment experiments on glutathione metabolism in a plant cell and its role in defense reactions. OTC = L-2-oxothiazolidine-4-carboxylic acid, SA = salicylic acid, ROS = reactive oxygen species.

A more detailed insight into the glutathione metabolism in ZYMV infected Styrian oil pumpkin plants was given by biochemical analysis of single tissues from the centre of the leaves, close to the main leaf vein. Interestingly, glutathione levels of intact dark-green leaf parts did not differ from uninfected samples, neither in upper nor in lower leaves (Zechmann et al. 2005). Moreover, immunocytochemical labeling of glutathione within single cells of the same dark-green

similar to what has been reported for other signaling compounds which interact with glutathione like SA (Murphy and Carr 2002) and cytokinins (Jameson and Clarke 2002).

Natural resistance mediated by glutathione: An antiviral strategy?

During the last two decades, modern genetic improvement programs of pumpkins have been carried out not only

to achieve resistance against numerous viruses, but also with the aim to increase the fruit yield, the number of seeds and the content of oil and antioxidants (Desbiez and Lecoq 1997; Lelley and Henglmüller 1999; Baranek et al. 2000; Bavec et al. 2002; Ivančič et al. 2004). Pathogen-derived resistance strategies using the expression of ZYMV genes in transgenic plants have been developed and appear promising. Although the genetic engineering strategies have been shown to be successful against viral diseases, transgenic plants have not been widely introduced till now, due to the registration problems related to biosafety issues. Nevertheless, the high biological variability of ZYMV justifies a careful evaluation of the deployment of genetic control strategies in order to increase their durability (Desbiez and Lecoq 1997). In contrast, natural resistance of plants against the viral diseases has gained increased international importance in agriculture in the last years and could be induced by treatments with various abiotic agents, known as plant inducers (Schneider and Ullrich 1994; Fodor et al. 1997; Gullner et al. 1999). Possibilities to increase cellular glutathione concentrations in plants are the use of different chemicals like herbicides (dinitroaniline, paraquat), resistance inducers [e.g. benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester, SA], artificial precursor of glutathione biosynthesis (OTC) and glutathione itself (Bolter et al. 1993; Görlach et al. 1996; Knörzer et al. 1999; Gullner and Dodge 2000; Will et al. 2001; Urbanek Krajnc et al. 2003a,b; Zechmann 2003; Hartmann et al. 2004; Urbanek Krajnc 2004; Müller et al. 2005).

Feeding experiments with glutathione showed that artificially elevated glutathione levels in different plant species correspond with suppression of virus induced symptoms (Gullner and Kömives 2001). The significance of glutathione during virus infection has also been proven in humans. It is well known that HIV seropositive individuals and humans infected with herpes simplex have reduced glutathione levels. The addition of exogenous glutathione was not only able to elevate the intracellular thiol levels, but also inhibited virus replication and progression of the disease (Palamara et al. 1995; Vossen et al. 1997).

Besides the positive roles of glutathione, it has been demonstrated recently that exogenously applied glutathione might be deleterious. Chromosomal defects, a decrease in the cell division rate and alterations in the ultrastructure were visible in spruce root meristem and callus cells by applying different concentrations of glutathione (Zellnig et al. 2000; Müller et al. 2001). The reported glutathione induced chromosomal aberrations and changed cell division rates lead to the assumption, that alterations in the cytoskeleton might be responsible for these effects (Urbanek Krajnc et al. 2003 a,b). An alternative way to prevent such damages caused by externally applied glutathione, but to keep the protection against pathogen infections by enhanced glutathione levels, is the artificially elevation of glutathione contents within the plants by glutathione precursors or chemicals, which are stimulating glutathione synthesis (Fodor et al. 1997; Gullner et al. 1999; Knörzer et al. 1999; Müller et al. 2005).

OTC, the, artificial L-cysteine precursor of glutathione biosynthesis is nowadays used to study the impact of increased glutathione concentrations on the protection of the

plant against pathogen attack. Elevated glutathione levels and suppressed necrotic lesions were reported on tobacco leaf discs, which were treated with 2 mM OTC two days prior the infection. In addition, the foliar virus concentration decreased in parallel with the lesion number, to 55 % of the control (Gullner et al. 1999). OTC was also applied in medical research in order to reduce human immunodeficiency virus (HIV) expression (Simon et al. 1994).

The injection of SA into tobacco leaves also resulted in increased glutathione levels (Fodor et al. 1997). Since an accumulation of SA is essential for the development of SAR it is thought that together with other antioxidants SA contributes to the appearance of resistance by increasing the capacity of the plant to scavenge ROS during a pathogen infection (Ryals et al. 1996; Fodor et al. 1997; Noctor 2006).

OTC and SA treatment experiments on Styrian oil pumpkin plants showed that both chemicals were rapidly taken up by the seedlings since both cotyledons and roots possessed a high affinity to assimilate these chemicals into thiols. The redox state of glutathione was not affected by the treatments (Urbanek Krajnc 2004). Increased glutathione levels were also determined in OTC and SA treated Styrian oil pumpkin callus cells and leaf protoplasts using different light and electron microscopic techniques (Zechmann 2003; Urbanek Krajnc 2004; Müller et al. 2005). Based on above described results one part of OTC and SA treated seedlings was further cultivated and mechanically infected with ZYMV to study the impacts of virus infection on symptom development. Alternatively, plants were regenerated from OTC treated callus tissues and controls, in order to find out whether physiological properties of *in vitro* plants could be useful for controlling plant diseases under field conditions. Exogenous applied OTC and SA induced delayed symptom development and provided an enhanced tolerance against ZYMV infection in Styrian oil pumpkin, by stimulating the synthesis, conversion and translocation of thiols during advanced ZYMV infection (Figure 3). A trend towards complete absence of ZYMV induced symptoms was observed on high percentage of the seedlings, which were treated with graduated concentration of OTC and SA. 31.2 % of 0.5 mM OTC showed no virus specific symptoms. 43.8 % of the 0.5 mM OTC treated plants responded with only minor symptoms in the form of yellowing of the young leaves. Severe symptoms were not determined. 1 mM OTC treated seed-derived plants were characterized by obvious reduction of symptom development. 68.8 % of them exhibited healthy leaves and normal growth forms with no visible symptoms. The other plants (25 %) revealed only light yellowing of the leaves (Table 1).

18.8 % of 0.5 mM SA pretreated plants were characterized as healthy. A high percentage of 0.5 mM SA treated plants (50 %) showed yellowing of the leaves and developed mild or severe mosaic symptoms. 37.5 % of 1 mM SA treated seedlings did not show any visible symptoms. Light yellowing of the leaves was observed on 43.8 % of the plants, but non of 1 mM SA treated plants showed severe mosaic symptoms. 3 mM SA treated seedlings were highly tolerant against ZYMV, since 68.8 % of 3 mM SA treated plants were characterized by a complete absence of ZYMV induced

symptoms on the leaves (Table 1) (Urbanek Krajnc 2004). Interestingly, virus particles were detected within leaves and roots of all OTC and SA treated plants by negative staining in the TEM (Table 1) (Zechmann 2003).

lus tissue and mature developed plants derived from somatic embryos, since a correlation between growth regulators and antioxidant levels has been reported in different plants (Berglund et al. 1993; Ohlsson and Berglund 2001; Bonfill et al. 2003). Biochemical analysis showed significant lower total glutathione levels among *in vitro* grown Styrian oil pump-

Table 1. Presence of virus particles and symptom characterization of ZYMV infected seed-derived and regenerated Styrian oil pumpkin plants. Two-week-old seedlings were treated with different concentrations of OTC (0 mM, 0.5 mM and 1 mM) and SA (0 mM, 0.5 mM, 1 mM and 3 mM) for 48 hours and were mechanically infected with ZYMV two weeks after transplantation to soil. Regenerated plants were treated with OTC (0 mM, 0.5 mM and 5 mM) for 14 days in callus stage and were infected with ZYMV five weeks after transplantation to soil. Values represent the amount of plants three weeks after ZYMV inoculation showing symptoms of ZYMV disease in % (n=16) (according to Urbanek Krajnc 2004).

ZYMV		Leaf					Root
		Virus	Symptoms ¹				Virus
			0	*	**	***	
Seed-derived plants	Controls	+	6.2	6.2	75.0	12.6	+
	0.5 mM OTC	+	31.2	43.8	25.0	0	+
	1 mM OTC	+	68.8	25.0	6.2	0	+
	0.5 mM SA	+	18.8	31.2	25.0	25.0	+
	1 mM SA	+	37.5	43.8	18.7	0	+
	3 mM SA	+	68.8	6.2	18.8	6.2	+
Regenerated plants	Controls	+	0	22.2	55.6	22.2	+
¹ Symptom	0.5 mM OTC	+	36.4	27.3	27.3	9.0	+
	5 mM OTC	+	66.6	16.7	16.7	0	+

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.

Plant regeneration via somatic embryogenesis in Styrian oil pumpkin: an object in virus research

Antiviral strategies based on the application of biotechnologies generally require regeneration of whole plants from a single cell or tissues in order to manipulate them to get desired compounds and physiological properties (Debeaujon and Branchard 1993; Ramachandra Rao and Ravishankar 2002). The use of *in vitro* culture techniques is of great importance in eliminating viruses. In contrast, callus tissues provide an alternative way for a long-term storage of plant viruses (Chen et al. 2003). Moreover, the physiological properties of *in vitro* cultivated plants enable them to react with suppressed symptoms on virus infection (Balazs et al. 1977; Sano et al. 1994; Dermastia and Ravnkar 1996; Jameson and Clarke 2002). Since the thin coated seed of the Styrian oil pumpkin is genetically recessive, character somatic embryogenesis from cotyledon explants would be important not only to obtain virus resistance by modulating the physiological properties of the plantlets, but also for the conservation of this local cultivar. Recently, the effects of different plant regulators on embryo yield, germination rate, maturation of somatic embryos and development of Styrian oil pumpkin plants were described. The accumulation of glutathione was studied during different developmental stages, including cal-

kin plantlets compared to seedlings grown in soil. When the plantlets were transferred into soil, they reached a normal size within a month and the glutathione concentration was comparable to seed-derived plants at the same developmental stage (Urbanek Krajnc et al. 2004).

The combination of growth regulators used for regeneration of Styrian oil pumpkin plants was not effective in plant improvement against viral disease later on. The first signs of virus symptoms in the form of yellowing were seen within seven days post inoculation, which was on average three days earlier when compared to ZYMV infected seed-derived plants. Three weeks after infection control plants developed typical ZYMV induced symptoms in the form of a strong yellow mosaic and dark green blisters (Urbanek Krajnc 2004).

When symptoms of infection were strongly expressed in untreated regenerated plants, OTC treatment in callus stage provided an enhanced tolerance of regenerated plants against ZYMV. 36.4 % of 0.5 mM OTC treated plants showed no visible symptoms three weeks after ZYMV infection. The other 54.6 % of the plants showed first signs of virus symptoms in the form of yellowing or first signs of mosaic patterns ten to 14 days after the inoculation. Only 9 % of regenerated plants, which were pretreated with 0.5 mM OTC in callus stage, developed severe virus symptoms.

Moreover, 66.6 % of 5 mM OTC pretreated plants were characterized by a complete absence of ZYMV induced symptoms on the leaves. The rest developed only minor yel-

lowing or first signs of mosaic. None of 5 mM OTC pretreated regenerated plants developed severe symptoms (Table 1) (Urbanek Krajnc 2004).

The regenerated plants, which were transferred to field, were all infected with virus from the neighboring pumpkin fields. However, only 20 % of the fruits, originated from 0.5 mM and 5 mM OTC treated plants showed deformation, the other showed no visible symptoms (Urbanek Krajnc 2004).

CONCLUSION AND FUTURE PERSPECTIVES

This review described the biochemical defense systems, which may provide sensitive tracers of virus impacts on plants. The concept that glutathione is one of the regulating chemicals in defense reactions during plant-virus interactions is now generally accepted (Gullner and Kömives 2001). In the present paper the glutathione concentrations and their efficiency in response to compatible ZYMV infection were evaluated within whole organs, single tissues and cells of Styrian oil pumpkin plants. Deficiency of total glutathione in ZYMV infected leaves demonstrates the need of the plant for cellular protection against ROS and shows up as externally visible symptoms. The question appeared whether glutathione deficiency of ZYMV infection is due to reduced synthesis rate secondary to shortage in cysteine availability, reduction in glutathione synthetase activity or reflects a rapid turnover of glutathione. Further investigations of glutathione precursors and enzymes involved in glutathione synthesis will help to unravel the complexity of glutathione dependent protective reactions.

The quantification of glutathione within single tissues and cells gave a detailed insight into the ZYMV induced changes in glutathione metabolism and represented that Styrian oil pumpkin plants respond on the same pathogen in different cell types with different capacity to resist oxidative stress. The determination of total glutathione and GSSG in early stages of virus infection will define more precisely the temporal aspect of glutathione mediated cellular protection against ZYMV.

Current studies demonstrated that exogenously applied OTC and SA are effective procedures to increase glutathione concentrations in plants (Fodor et al. 1997; Gullner et al. 1999; Knörzer et al. 1999). Both chemicals induced delayed symptom development and provided an enhanced tolerance against ZYMV infection stimulating the synthesis, conversion and translocation of thiols during advanced symptom appearance (Urbanek Krajnc 2004). The present treatment experiments could aid in the development of new practices aimed at the protection of Styrian oil pumpkin plants against severe epidemics of ZYMV disease without changing the genetic information and, in turn, save the Styrian oil pumpkin industry from severe crop losses in the future.

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