FINAL REPORT

on the research project NKFIH 124131 entitled "Reactive Sulfur Species (RSS) in fungus and virus-infected plants - new perspectives for the use of sulfur to control plants diseases"

Dr. Gábor Gullner

Theoretical background

Sulfur (S) metabolites are involved in plant disease resistance. Latest research in that field led to the development of the concept of sulfur induced resistance (SIR) (Bloem et al., 2015), which is also knowns as sulfur-enhanced defense (SED) (Kruse et al., 2007). The mechanisms of this sulfur-induced resistance (SIR) are, however, not yet known. The inorganic sulfate anion taken up by plants from the soil is converted to sulfide anion through an intermediate sulfite form. The sulfur atom is ultimately incorporated into cysteine, the first organic molecule carrying reduced sulfur (Leustek et al., 2000). Cysteine serves as the precursor of a wide variety of antimicrobial or antioxidative thiol compounds such as defensins, glucosinolates, glutathione, glutathione S-transferases, phytoalexins, S-containing volatiles and thionins (Rausch and Wachter, 2005). Currently the Reactive Sulfur Species (RSS) are in the focus of interest of numerous research groups due to their participation in cellular signaling and regulatory processes. RSS are a diverse group of redox active sulfur containing compounds that are capable of either oxidize or reduce biomolecules under physiological conditions (Gruhlke and Slusarenko, 2012). Two RSS, hydrogen sulfide (H_2S) and sodium sulfite have been recently shown to play critical roles in plant disease resistance (Shi et al., 2015). The connection between H_2S and plant biotic stress resistance has been revealed but the underlying mechanisms are still largely unknown. Beside H2S, the sulfite anion has recently been also associated with plant disease resistance as a possible signal molecule (Giraud et al., 2012). The present project was aimed at the molecular characterization of plant reactions activated by hydrogen sulfide and sulfite anion. For this purpose we selected sodium sulfide and sodium sulfite treatments in order to activate the cysteine- and glutathione-dependent plant defense processes.

Initial experiments

At the beginning of the project we tested the phytotoxicity of aquous solutions of sodium sulfide (Na_2S) and sodium sulfite (Na_2SO_3) containing Tween-20 as detergent in a wide concentration range by spraying them on middle leaves of two-month-old pepper plants as well as on leaves of two-week-old barley seedlings. The two tested compounds showed no apparent phytotoxic effects up to the concentration of 10 mM, which was around their solubility limits.

In the following experiments we treated middle leaves of two-month-old pepper plants (*Capsicum annuum* L.) by spraying them with aquous solutions of sodium sulfide and sodium sulfite containing Tween-20 as detergent. We analyzed the endogenous cysteine and glutathione levels of the treated and control (Tween-20 treated) pepper leaves after different time points with HPLC and fluorescent detection. Both the Na_2S and Na_2SO_3 treatments significantly increased the cysteine levels and to a lesser extent also that of glutatione depending on the concentration of Na_2S and Na_2SO_3 solutions and on the time period of treatment (Figure 1).

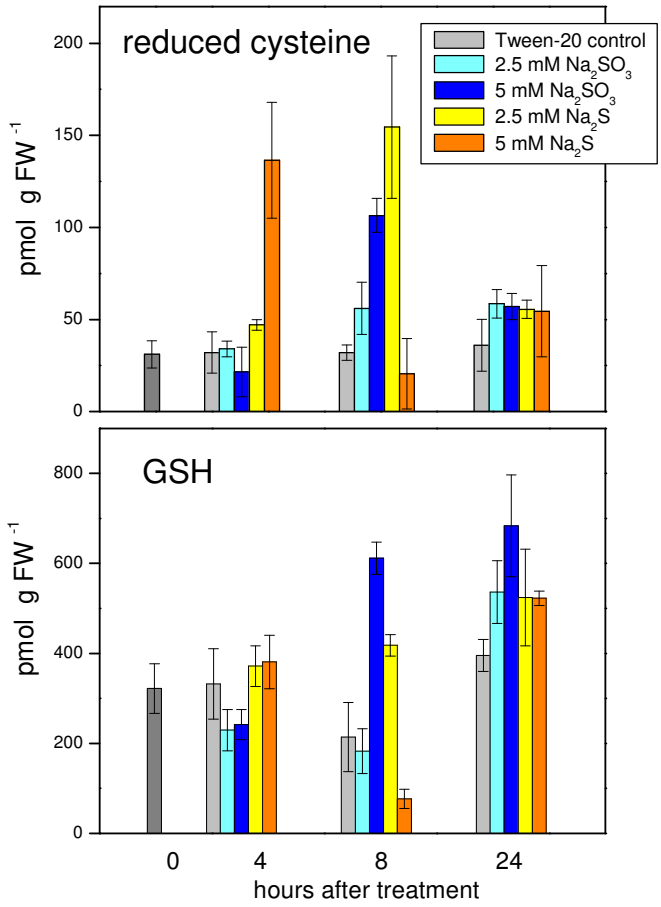

Figure 1. Changes in the cysteine and glutathione content of pepper leaves following treatments with aquous solutions of sodium sulfide (Na_2S) and sodium sulfite (Na_2SO_3) containing Tween-20 as detergent. Mean values of three independent experiments ± SD are shown.

We tested the interaction between *Nicotiana benthamiana* and the hemibiotrophic fungal pathogen *Cercospora nicotianae*. This fungus can be conveniently used under laboratory conditions therefore we planned to involve this interaction into the present project. Our inoculation studies showed that *C. nicotianae* more effectively colonized *N. benthamiana* than its conventional host, *Nicotiana tabacum* (Fodor et al., 2018). Although the *N. benthamiana–C. nicotianae* host–pathogen interaction seemed to be a prospective pathosystem, finally it was not used during the present project, because we selected the economically more important barley - barley powdery mildew interaction.

Investigation of pepper - tobamovirus interactions

Pepper (*Capsicum annuum* L. cultivar TL 1791) plants containing the L^3 resistance gene were inoculated with *Obuda pepper virus* (ObPV) and with *Pepper mild mottle virus* (PMMoV). Both viruses are positive-strand RNA viruses and they belong to the genus Tobamovirus (Tóbiás et al., 1989). ObPV inoculation resulted in the appearance of necrotic lesions due to L^3-mediated resistance (incompatible interaction), while PMMoV caused only very slight chlorotic symptoms in inoculated leaves (compatible interaction) (Tóbiás et al., 1989). We carried out pre-treatments on pepper leaves with aquous solutions of sodium sulfide and sodium sulfite

containing Tween-20 as detergent, which were followed by inoculations with two different tobamoviruses. The sulfide or sulfite supply did not influence significantly the visible symptoms of virus inoculations on pepper leaves (data not shown).

Total RNA was purified from the infected and control leaves, and the expression of coat protein (CP) genes measured by RT-PCR method. Pretreament of pepper leaves with sodium sulfide (Na_2S) and sodium sulfite (Na_2SO_3) did not modify significantly the expression of *CP* genes showing that these reactive sulfur species do not increase the antiviral ressitance of pepper leaves (Figure 2).

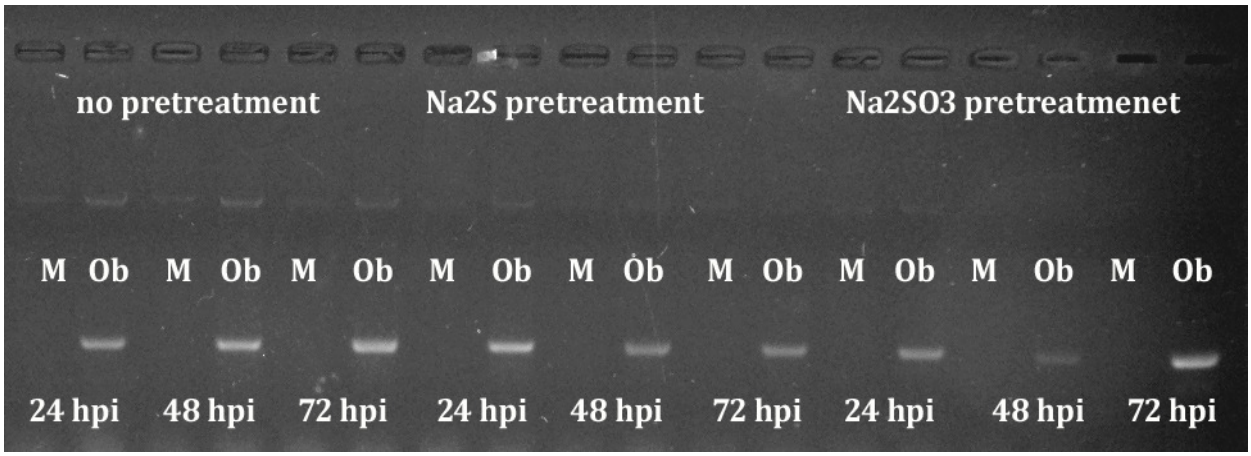

Figure 2. Changes in the expression of coat protein (*CP*) gene of *Obuda pepper virus* (ObPV) in sodium sulfide (Na_2S) and sodium sulfite (Na_2SO_3) pre-treated and ObPV-inoculated pepper leaves at different time points following inoculation as detected by RT-PCR. Forward primer (5'-3' direction): AAGACTGTTCCCACTAGGACC, reverse primer (5'-3' direction): TTGGACACCGTTTCGAAAG. PCR conditions: annealing at 60 °C, 28 cycles. Abbreviations: M: mock-inoculated (virus-free) pepper leaves; Ob: *Obuda pepper virus* (ObPV) inoculated leaves; hpi: hours post inoculation.

Gene expression studies in pepper - tobamovirus interactions

We wanted to explore the role of cysteine biosynthetic enzymes in the defense processes of virus inoculated plants. For this purpose first we identified in the NCBI GenBank database those pepper genes that encode enzymes participating in the biosynthesis of cysteine. Three ATP-sulfurylase, four adenosine 5'-phosphosulfate reductase (also called 5'-adenylylsulfate reductase), one sulfite reductase, one sulfite oxidase, seven O-acetylserine-(thiol)-lyase (OAS-TL) and five serine-acetyltransferase (SAT) were identified. To learn more about the role of cysteine biosynthesis in virus-infected plants, we analyzed the expression levels of several cysteine biosynthetic genes in extracts from ObPV- and PMMoV-inoculated pepper leaves with RT-PCR by using specific primer pairs. Leaf samples were taken at 4, 8, 12, 24 and 48 hours after virus inoculations. We investigated the expression levels of four 5'-adenylylsulfate reductase (APR 1-4), a sulfite oxidase, a sulfite reductase, and four OAS-TL genes. The expression of these gene did not change significantly following ObPV or PMMoV inoculations (data not shown).

The biosynthesis of the defense hormone ethylene is evidently reliant on sulfur bioavalability and connected to methionine metabolism. The enzyme 1-aminocyclopropane-1-carboxylic acid sythase (ACS) is the key regulatory factor involved in the biosynthesis of ethylene. Therefore we investigated the expression of ACS genes in pepper leaves inoculated with ObPV and PMMoV. First we carried out a search for pepper ACS genes in the NCBI GenBank database and identified eight distinct ACS genes. RT-PCR studies showed that the expression of two ACS genes were significantly up-regulated 24 and 48 hours after ObPV inoculation whereas PMMoV inoculation had no effect on the expression of these genes. On the other hand, the expression of six ACS genes did not change following neither ObPV nor PMMoV inoculation. These results showed that two ACS genes can play a role in the ObPV-resistance of pepper plants (data not shown).

Fatty acid desaturases in pepper - tobamovirus interactions

We were also interested in the link between sulfur amino acids (methionine, cysteine, homocysteine and taurine) and lipid metabolism. Therefore we studied the role of fatty acid desaturase (*FAD*) genes in pepper leaves inoculated with ObPV and PMMoV. We carried out a search for pepper FAD genes in the NCBI GenBank database and identified 27 distinct FAD genes. Specific primer pairs were designed for 17 representative FAD genes. RT-PCR and quantitative real-time RT-qPCR experiments were carried out to study the changes in the expressions of these 17 FAD genes in pepper leaves inoculated with ObPV and PMMoV. ObPV inoculation led to a massive induction of five FAD genes, which were not activated by PMMoV (Figure 3).

According to *in silico* predictions the strongly ObPV-inducible FAD genes encode omega-6-desaturase enzymes located in the endoplasmic reticulum of host plant cells. Such FAD enzymes catalyze the conversion of oleic acid (18:1) to linoleic acid (18:2). One further FAD gene was specifically induced only by PMMoV whereas one other FAD gene was activated by both viruses. Ten FAD genes were not activated by any virus infections. We suppose that the enzymes encoded by the ObPV-inducible FAD genes contribute to virus resistance by modifying intracellular plant membranes, which are critical for virus replication (Balogh et al., 2019 and 2020).

In separate experiments we studied the fatty acid composition of pepper leaves following ObPV and PMMoV inoculations by gas chromatography–mass spectrometry (GC-MS) preceded by a chemical derivatization of fatty acids. The following fatty acids were quantitatively analyzed: hexadecanoic acid (16:0), hexadecenoic acid (16:1), 7,10-hexadecadienoic acid (16:2), 7,10,13-hexadecatrienoic acid (16:3), octadecanoic acid (18:0), 9-octadecenoic acid (18:1), 9,12-octadecadienoic acid (18:2) and 9,12,15-octadecatrienoic acid (18:3). In uninfected pepper leaves we identified linolenic acid (18:3), palmitic acid (16:0), linoleic acid (18:2) and hexadecatrienoic acid (16:3) as the main fatty acid components. ObPV inoculation led to a slight (1.4-fold) but significant rise of linoleic acid (18:2) content in pepper leaves at 72 hours post-inoculation. PMMoV inoculation did not exert any effect on the linoleic acid content. The levels of all other fatty acids did not change significantly following neither ObPV- nor PMMoV-inoculation. These GC-MS observations supported our earlier results on FAD gene expression levels (Balogh et al., 2019 and 2020).

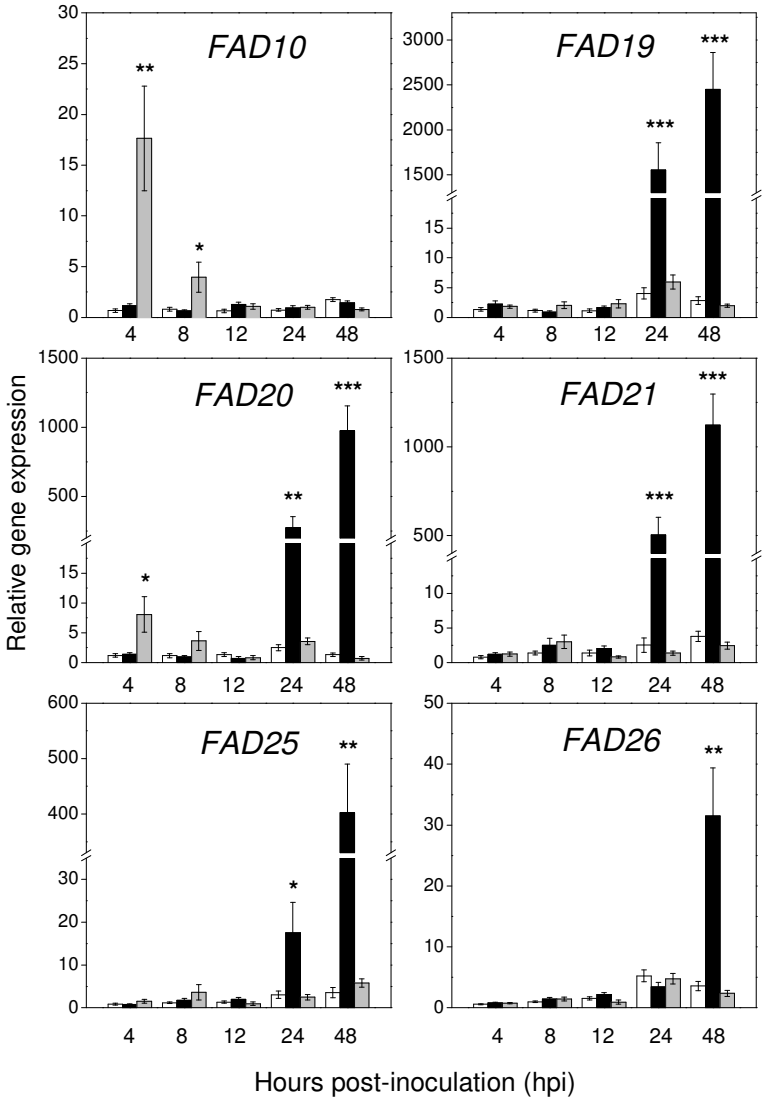

Figure 3. Relative expression of six FAD genes in pepper leaves following inoculation with *Obuda pepper virus* (ObPV) or *Pepper mild mottle virus* (PMMoV) or after mock-inoculation as determined by real-time RT-qPCR with gene-specific primer pairs. The expression of UBI-3 gene was used as control. Open, black and gray columns represent mock- , ObPV- and PMMoV-inoculated leaves, respectively. Mean values of three independent experiments are shown ± SD. The symbols *, ** and *** show significant differences between mock- and virus-inoculated plants at P < 5%, < 1% and < 0.1%, respectively (Balogh et al., 2020).

RNA-Seq studies of pepper - tobamovirus interactions

The comparison of gene expression patterns between an incompatible and a compatible plant-virus interaction can provide valuable information about host genes that have key roles in resistance. Therefore we identified those pepper genes that were rapidly and robustly activated only in the incompatible interaction, because these genes may have crucial roles in the antiviral defense of pepper. To learn more about the role of sulfate and cysteine metabolism in the virus resistance mechanism of pepper, we carried out transcriptome-wide RNA-sequencing experiments in collaboration with an external sequencing company (Xenovea Ltd., Szeged).

We isolated total RNA extracts from the ObPV-, PMMoV- and mock-inoculated pepper leaves at 4, 8, 24 and 48 hours post-inoculation (hpi), so we obtained 12 RNA libraries. First, we investigated the multiplication of ObPV and PMMoV in the inoculated pepper leaves by measuring the amount of mRNAs encoding viral movement proteins (MP) and coat-proteins (CP) in the total leaf RNA extracts by quantitative, real-time RT-PCR. Generally, the accumulation of PMMoV was markedly stronger than that of ObPV in spite of the absence of any symptoms on PMMoV-inoculated leaves (Figure 4) (Kalapos et al., 2021).

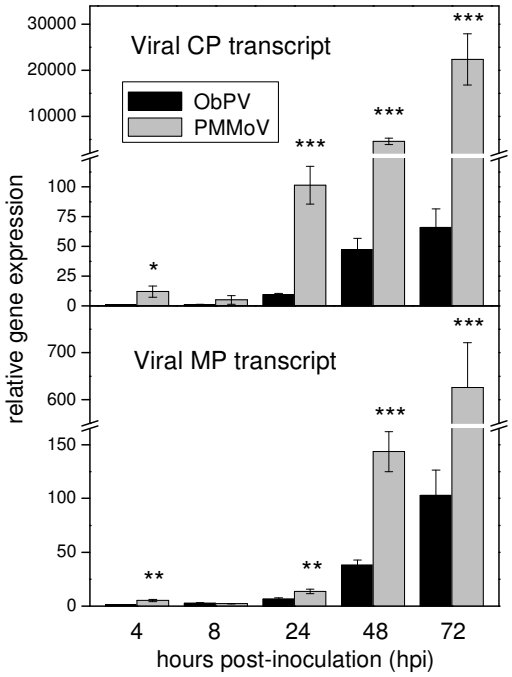

Figure 4. Multiplication of *Obuda pepper virus* (ObPV) and *Pepper mild mottle virus* (PMMoV) in the inoculated pepper leaves at different time points following the inoculations. The amount of mRNAs encoding viral movement proteins (MP) and coat proteins (CP) of ObPV and PMMoV was detected by real-time RT-qPCR. Mean values of three independent experiments are shown ± SD. The symbols *, ** and *** show significant differences between ObPV- and PMMoV-inoculated plants at $P < 5\%$, $< 1\%$ and $< 0.1\%$, respectively (Kalapos et al., 2021).

The RNA-Seq analysis resulted in a massive amount of qualitative and quantitative data about the gene expression changes in virus infected pepper leaves. In each RNA sample, the expression of approx. 22,000 genes was successfully detected. Both virus inoculations led to the significant up- or down-regulation of several hundreds of genes. Generally, the ObPV-inoculation exerted a stronger effect on the gene expression profile of pepper leaves than PMMoV. The bioinformatical KEGG-analysis of biochemical pathways indicated the strong activation of glutathione S-transferase dependent detoxification reactions by ObPV inoculation (Figure 5) (Kalapos et al., 2021).

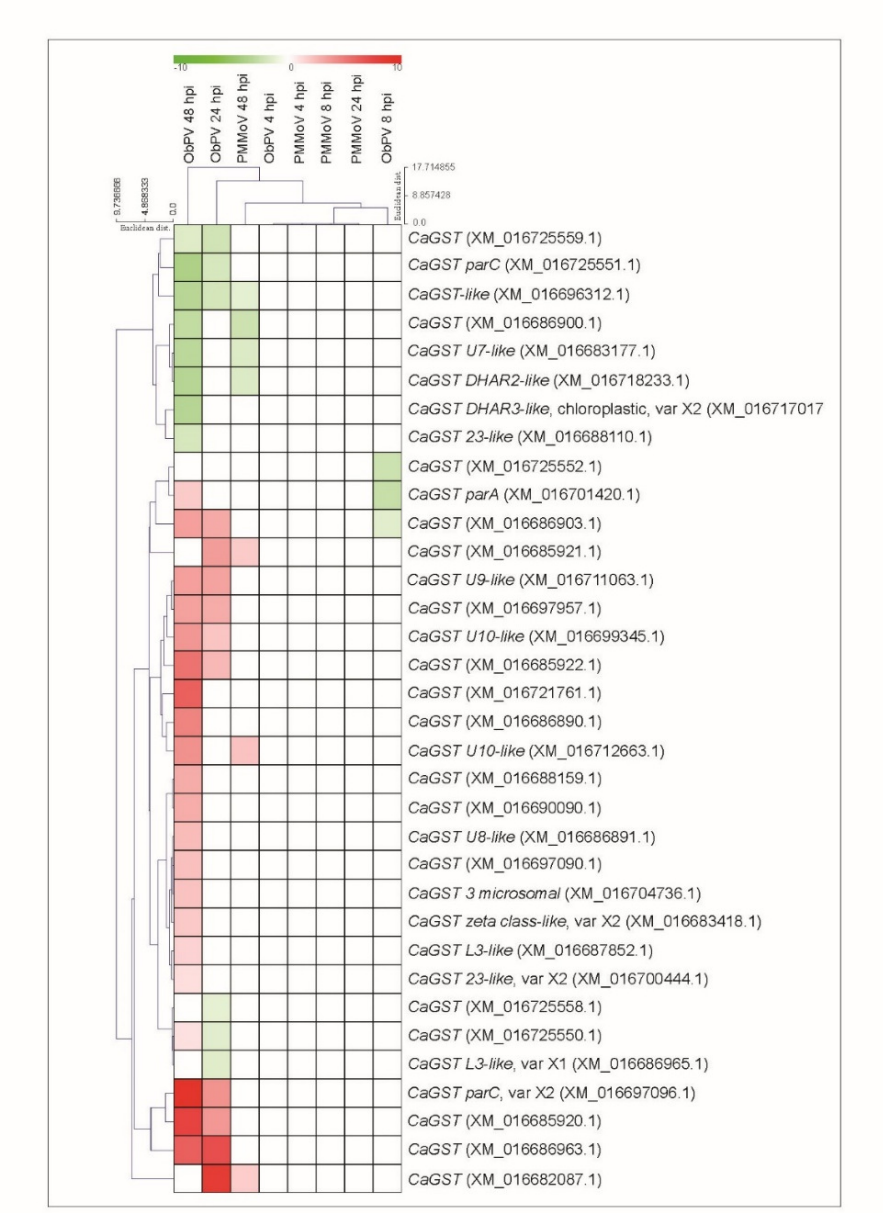

Figure 5. Heat-map representing changes in the expression of 34 glutathione S-transferase (*GST*) genes in pepper leaves inoculated with *Obuda pepper virus* (ObPV) and *Pepper mild mottle virus* (PMMoV) at 4, 8, 24 and 48 hours post-inoculation (hpi). Euclidean distance based hierarchical clustering was performed with average-linkage both among each of the GSTs and samples.

Genes participating in the metabolism of sulfur-containing amino acids (cysteine and methionine) were also markedly up-regulated, such as cysteine synthase genes, homocysteine S-methyltransferases, glutamate-cysteine ligase, and glutathione synthetase (Table 1).

The biosynthesis of the defense hormone ethylene is evidently reliant on S bioavailability and connected to methionine metabolism. Our RNA-Seq experiments also indicated the massive up-regulation of genes encoding 1-aminocyclopropane-1-carboxylate oxidases, 1-aminocyclopropane-1-carboxylate synthases and ethylene-responsive transcription factors in

ObPV				PMMoV				Description	Gene ID
4 hpi	8 hpi	24 hpi	48 hpi	4 hpi	8 hpi	24 hpi	48 hpi		
								sulfate transporters	
		1.84						sulfate transporter 1.2-like, transcript variant X6	LOC107851042
	-1.31	2.08	4.87					sulfate transporter 3.1-like, transcript variant X2	LOC107863792
		-2.43	-3.34					low affinity sulfate transporter 3	LOC107867559
		-1.66	-3.69					sulfate transporter 3.1-like	LOC107875280
								cysteine biosynthesis	
		1.48						5'-adenylylsulfate reductase 1, chloroplastic	LOC107859594
			2.13					adenylyl-sulfate kinase 3-like	LOC107848526
		1.89						serine acetyltransferase 1, chloroplastic-like	LOC107878792
		-2.49	-3.89	-1.40				serine acetyltransferase 1, chloroplastic-like	LOC107845759
			1.80					serine acetyltransferase 5	LOC107840956
		3.45	4.03					cysteine synthase-like, transcript variant X3	LOC107839482
		2.69	1.80				-1.37	bifunctional L-3-cyanoalanine synthase/cysteine synthase 2, mitochondrial, transcript variant X1	LOC107839203
								cysteine metabolism	
		-1.32					-1.34	cysteine desulfurase 1, chloroplastic	LOC107871353
								methionine biosynthesis	
			1.58					bifunctional aspartokinase/homoserine dehydrogenase	LOC107848347
			-2.94					bifunctional aspartokinase/homoserine dehydrogenase 1, chloroplastic-like	LOC107879648
							-1.17	cystathionine gamma-synthase 1, chloroplastic-like	LOC107858737
								methionine cycle	
			4.12					homocysteine S-methyltransferase 2	LOC107864144
		2.22	1.75					S-adenosylmethionine synthase 3-like	LOC107843579
			-4.34					putative S-adenosyl-L-methionine-dependent methyltransferase	LOC107867000
							-1.09	adenosylhomocysteinase	LOC107864322
			1.77					S-adenosylmethionine decarboxylase proenzyme	LOC107847415
			1.90					probable S-adenosylmethionine carrier 2, chloroplastic, transcript variant X3	LOC107851376
			1.99					spermine synthase-like	LOC107855970
								methionine sulfoxide reductases	
		2.06	2.66					peptide methionine sulfoxide reductase	LOC107862630
			-2.18					peptide methionine sulfoxide reductase A1-like	LOC107863514
			-1.09					peptide methionine sulfoxide reductase B1, chloroplastic	LOC107878558
			-3.25					peptide methionine sulfoxide reductase B5-like	LOC107879910
								glutathione biosynthesis	
		1.22						glutamate--cysteine ligase, chloroplastic, transcript variant X1	LOC107865075
		2.16	1.74					glutathione synthetase, chloroplastic, transcript variant X1	LOC107839536
								glutathione metabolism	
				-1.55				putative gamma-glutamylcyclotransferase At3g02910	LOC107839716
1.76			2.21	1.44				putative gamma-glutamylcyclotransferase At3g02910	LOC107866072
		-1.50						probable lactoylglutathione lyase, chloroplastic, transcript variant X2	LOC107841018
		-1.29						putative lactoylglutathione lyase, transcript variant X1	LOC107859593
			1.72					glutathione reductase, chloroplastic	LOC107864303
			1.74					glutathionyl-hydroquinone reductase YqjG-like, transcript variant X2	LOC107858940
			-2.45					glutathionyl-hydroquinone reductase YqjG	LOC107875310
								glutathione peroxidases	
			-2.57					probable glutathione peroxidase 4, transcript variant X2	LOC107842504
			-1.93					probable glutathione peroxidase 5, transcript variant X2	LOC107842555
			-3.57				-1.52	probable phospholipid hydroperoxide glutathione peroxidase	LOC107848506
			2.24					probable phospholipid hydroperoxide glutathione peroxidase	LOC107864929
								glutaredoxins	
			-1.63					bifunctional monothiol glutaredoxin-S16, chloroplastic	LOC107842530
			-3.14					monothiol glutaredoxin-S3-like	LOC107857140
			-2.97					monothiol glutaredoxin-S10-like	LOC107857141
			1.25					monothiol glutaredoxin-S6-like, transcript variant X1	LOC107859924
			-1.36					glutaredoxin-C13	LOC107866398
		2.26						monothiol glutaredoxin-S2-like	LOC107866400
			-6.86					monothiol glutaredoxin-S6-like	LOC107872589
			-3.62					monothiol glutaredoxin-S3-like	LOC107874941
			-4.79					glutaredoxin-C13-like	LOC107875177
		-1.40						monothiol glutaredoxin-S15, mitochondrial	LOC107876039
1.67			2.43					glutaredoxin-C9-like	LOC107878394

Table 1. Changes in the expression of sulfur metabolism genes detected in pepper leaves inoculated with *Obuda pepper virus* (ObPV) or *Pepper mild mottle virus* (PMMoV) at 4, 8, 24 and 48 hours post-inoculation (hpi) detected by RNA-Seq. Significant gene expression values were filtered by two treshold values: $p \leq 0.01$ and $-1 \geq \log2$ fold change (FC) ≥ 1 (Kalapos et al., 2021).

pepper leaves inoculated by ObPV. Already well-known effects of virus inoculations were also confirmed, such us the strong the activation of the phenylpropanoid pathway (phenylalanine ammonia-lyase-like genes) and particularly the activation of terpenoid biosynthesis (hydroxymethylglutaryl-CoA synthase-like genes and 3-hydroxy-3-methylglutaryl-coenzyme A reductase genes).

KEGG-analysis of biochemical pathways indicated the strong up-regulation of terpenoid biosynthesis, endocytosis, ABC-transporters, MAPK-signaling, phosphatidylinositol signaling, ethylene responses, RNA and protein degradation, protein ubiquitination, protein processing in the endoplasmic reticulum, cysteine and methionine metabolism, glutathione metabolism, the phenylpropanoid pathway, the citrate cycle and the fatty acid degradation as well as the down-regulation of the pentose phosphate pathway, the photosynthetic processes and carbon fixation in the ObPV-inoculated pepper leaves. In contrast to ObPV, PMMoV influenced these processes only negligibly or not at all.

We carried out the validation of RNA-Seq data by real-time PCR studies. The expression of twelve pepper genes has been examined in the ObPV- , PMMoV- and mock-inoculated pepper leaves at different time points following the inoculations. Generally, we found good correlations between the RNA-Seq and real-time PCR results (Kalapos et al., 2021).

Functional analysis of selected virus-inducible pepper genes

During our RNA-Seq investigations we identified numerous pepper genes that were markedly induced by tobamovirus inoculations. However, in most cases the exact function of the proteins encoded by these genes is unknown. Therefore we started the functional analysis of three pepper genes that were particularly strongly activated in the incompatible pepper-ObPV interaction. Genes encoding an ethylene reponsive transcription factor, a WRKY transcription factor and a glutahione S-transferase were selected. These genes were only negligibly induced in the compatible pepper-PMMoV interaction.

We used the 'Gateway' system developped by Invitrogen to the transient overexpression of these pepper genes in *Nicotiana benthamiana* plants. First, we constructed Gateway entry vectors and then cloning vectors carrying the above pepper genes. The vectors were multiplied in *E. coli* colonies, then introduced into a special *Agrobacterium tumefaciens* strain. Then we prepared genetically modified *N. benthamiana* plants that transiently overexpressed the ObPV-inducible pepper genes. The transient overexpressor *N. benthamiana* plants were created by the injection of *Agrobacterium* cultures carrying the pepper genes into the leaves of mature *N. benthamiana* plants. We determined the temporal profiles of the expressions of pepper genes by real-time quantitative PCR method in the presence or absence of a gene-silencing suppressor. We revealed the the applied gene-silencing suppressor construct markedly increased and prolonged the expression of pepper genes, but these effect significantly varied among the three pepper genes (data not shown).

Investigation of tobacco - tobacco mosaic virus interactions

To learn more about the role of sulfur-treatment in plant disease resistance, we investigated the effects of exogenously applied glutathione (GSH) on the *Tobacco mosaic virus* (TMV)-resistance of Xanthi NN tobacco as well as on its transgenic NahG line. In NahG plants salicylic

acid (SA) can not accumulate following TMV infection, because these plants express the bacterial *nahG* gene encoding salicylate hydroxylase that converts SA to catechol. The external application of the synthetic glutathione precursor R-2-oxo-4-thiazolidine-carboxylic acid (OTC) was also investigated. Infiltration of exogenous GSH or OTC into tobacco leaves resulted in a substantial rise of bound SA and to a lesser extent of free SA levels in tobacco, especially following TMV infection. We demonstrated that in SA-deficient NahG tobacco the treatment with exogenous GSH or OTC confers enhanced resistance to TMV manifested as both reduced symptoms (i.e. suppression of hypersensitive-type localized necrosis) and lower virus titers. The beneficial effects of elevated GSH on TMV resistance was markedly stronger in NahG than in Xanthi NN leaves. Significant increases in expression of pathogenesis related genes (NtPR-1a and NtPRB-1b) were observed in TMV-inoculated leaves. These results showed that elevated levels of glutathione in TMV-infected tobacco can compensate for SA deficiency to maintain virus resistance (Künstler et al., 2019) .

Effects of elevated glutathione level on powdery mildew resistance in tobacco

The effects of elevated glutathione levels on defense responses to powdery mildew (*Euoidium longipes*) were also investigated in Xanthi NN tobacco as well as in its SA-deficient NahG line. Aqueous solutions of GSH and OTC were injected into leaves of tobacco plants 3 hours before powdery mildew inoculation. SA-deficient NahG tobacco was hyper-susceptible to *E. longipes*, as judged by significantly more severe powdery mildew symptoms and enhanced pathogen accumulation. Strikingly, elevation of GSH levels in SA-deficient NahG tobacco restored susceptibility to *E. longipes* to the extent seen in wild-type plants. These results demonstrated that artificial elevation of glutathione content can significantly reduce the susceptibility to powdery mildew in SA-deficient tobacco (Künstler et al., 2020). In connection with the antioxidative role of glutathione, we also investigated the contribution of different pathways leading to the formation of reactive oxygen species to the overall oxidative stress phenomenon (Kámán-Tóth et al., 2019).

Investigation of barley - barley powdery mildew interactions

We carried out experiments also with barley (*Hordeum vulgare* L.) plants in order to test the effects of sulfur treatments on the disease symptoms following artificial inoculations with the obligate biotrophic fungal pathogen barley powdery mildew (*Blumeria graminis* f.sp. *hordei*). In these experiments we pre-treated two-week-old barley seedlings by spraying them with varying concentrations of sodium sulfide and sodium sulfite containing Tween-20 as detergent. Inoculations with powdery mildew fungus were carried out 1 day after sulfur pre-treatment. The chemical treatments exerted no influence on the visible disease symptoms (leaf area covered by fungal colonies). Fungal inoculations were carried out 2 hours after sulfur pre-treatments but these treatments were inefficient as well. To test the potential curative effect of sulfur treatments, barley seedlings were firstly inoculated with powdery mildew and 6 days later treated with aquous solutions of sodium sulfide and sodium sulfite. The chemical treatments had no effect on the visible disease symptoms (data not shown).

Later, we analyzed the physiological changes activated upon powdery mildew inoculation of the susceptible barley cultivar Ingrid and its near-isogenic lines carrying various resistant genes (*Mla*, *Mlg* and *mlo*). We focused on hormonal changes in the fungus-inoculated barley leaves that were measured by an HPLC-MS method. The investigated hormones were divided into four groups; cytokinins, auxins, gibberellins and “stress hormones”, which involved abscisic

acid (ABA), jasmonic acid (JA) and salicylic acid (SA). In healthy leaves of the near-isogenic barley lines harboring various resistance genes the hormone levels were most often similar to susceptible Ingrid leaves, or sometimes slightly reduced. However, after inoculation, in most the cases the amount of individual hormones increased or did not change.

Among the cytokinins, the amount of cis-zeatin riboside (cytokinin transport form) significantly increased in inoculated susceptible Ingrid leaves, and in the hypersensitive Mla plants. The amount of N6-isopentenyladenine (IPA, precursor form) significantly increased only in the inoculated leaves of Mla.

Changes in auxin homeostasis after powdery mildew inoculation were quite extended and concerned almost all tested lines. Powdery mildew inoculation significantly elevated the contents of indole-3-acetic acid (IAA, active form), indole-3-acetic acid methyl ester (MeIAA, precursor form) and oxindole-3-acetic acid (oxIAA, degradation form) in Ingrid and Mla lines. Elevated IAA level is considered as part of defence mechanism as auxins have a fungistatic effect. Intriguingly, we observed an extremely increased content of indole-3-acetyl-L-aspartic acid (IAAsp, degradation form) in the inoculated Mla line (Figure 6).

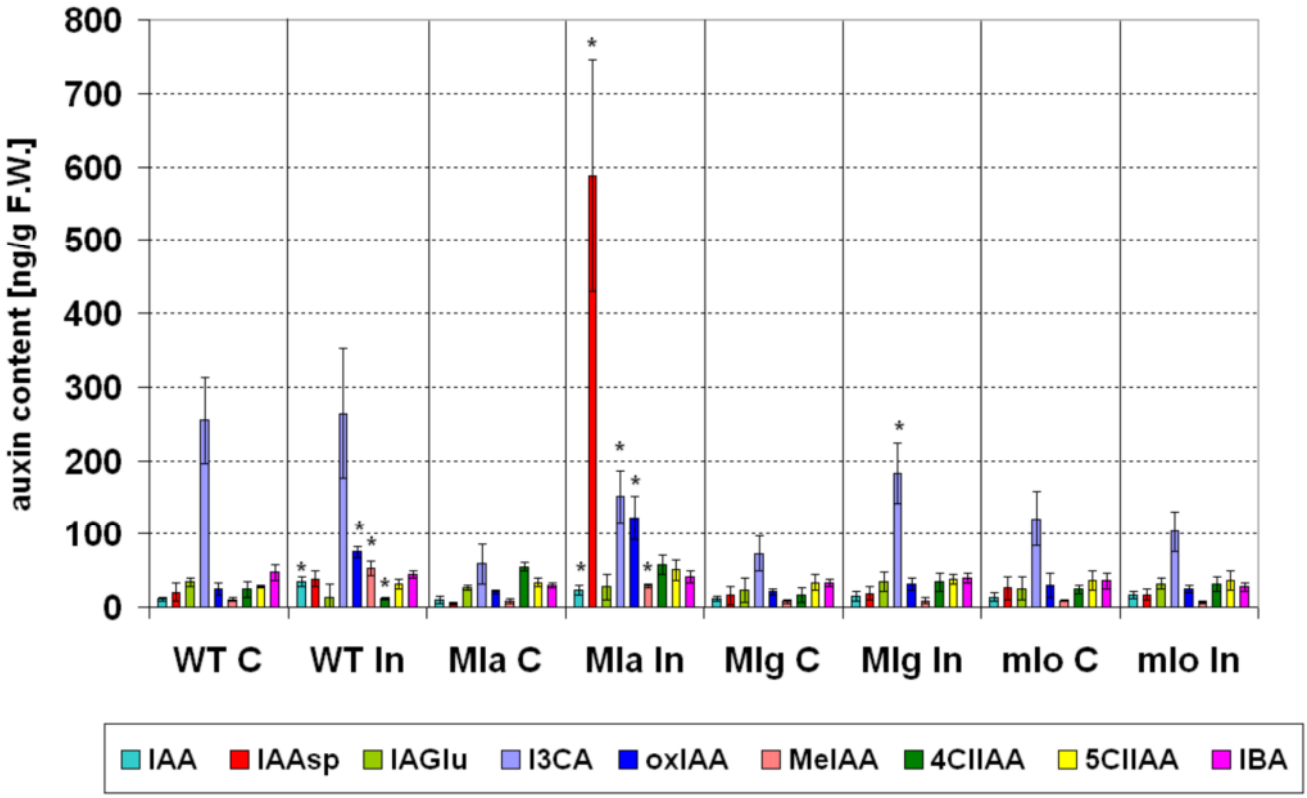

Figure 6. Changes in the contents of nine auxins in leaves of the susceptible barley cultivar Ingrid and its three near-isogenic lines Mla, Mlg, and mlo at seven days following powdery mildew inoculation (7 dpi). Abbreviations: C, control; Inoc, powdery mildew inoculated; WT, wild type cultivar Ingrid. Investigated hormones: indole-3-acetic acid (IAA), indole-3-carboxylic acid (I3CA), 4-chloroindole-3-acetic acid (4ClIAA), 5-chloroindole-3-acetic acid (5ClIAA), indole-3-acetyl-L-glutamic acid (IAGlu), indole-3-acetic acid methyl ester (MeIAA), indole-3-acetyl-L-aspartic acid (IAAsp), oxindole-3-acetic acid (oxIAA), and indole-3-butyric acid (IBA) (Saja et al., 2020).

The increased IAAsp concentration might be pathogen-stimulated in order to weaken the antifungal defense in this resistant genotype. Powdery mildew inoculation of Mla and Mlg lines that showed hypersensitive reaction led to significantly elevated levels of indole-3-carboxylic acid (I3CA, auxin degradation form). I3CA level was generally lower in resistant lines Mla, Mlg and mlo than in Ingrid (both in control and inoculated plants) (Figure 6).

The profile of gibberellins was generally very similar among our barley lines with only a small exception (Mla) and this profile did not change even in the susceptible Ingrid after powdery mildew infection. Upon fungal inoculation, a significant increase of gibberellin A1 (GA1, active form) level and a slight but significant elevation of gibberellin A6 (GA6, active form) level were observed only in Mla leaves. Presumably gibberellins do not play a pivotal role in the powdery mildew resistance of barley.

Powdery mildew inoculation caused strong changes in stress hormone levels of barley lines. Inoculated leaves of Mla plants showed significantly increased levels of JA, SA, ABA (active forms), while inoculated Mlg leaves had significantly elevated JA and SA contents in comparison to healthy ones. Interestingly, in susceptible Ingrid leaves the JA content significantly decreased after inoculation in comparison to non-inoculated control.

Our data on hormonal changes in powdery mildew inoculated barley lines confirmed that not only SA, JA or ethylene, but other hormones and their derivatives can play important roles in disease resistance or susceptibility to a biotrophic pathogen (Saja et al., 2020).

Defence responses triggered by Blumeria graminis f.sp. hordei in non-host wheat genotypes

We investigated also the barley powdery mildew (*B. graminis* f.sp. *hordei*)-triggered defense responses in seven wheat genotypes, and the induction of resistance by this non-host pathogen to a second, challenge infection with leaf rust, *Puccinia triticina* Eriks (formerly known as *Puccinia recondita* f.sp. *tritici*). Ascorbate peroxidase, glutathione S-transferase and catalase enzyme activities were markedly augmented in wheat leaves 2 days after powdery mildew infection. These responses showed the importance of antioxidative enzymatic reactions in the development of non-host resistance in wheat leaves (Barna et al., 2022).

RNA-Seq studies of barley - barley powdery mildew interactions

To identify significant defense genes and pathways during barley - barley powdery mildew interactions we carried out the transcriptome-wide Illumina RNA-Seq analyses of total RNA extracts prepared from powdery mildew infected and control barley leaves with the help of an external company. For these studies we applied the barley cultivar Ingrid (susceptible to powdery mildew) and its near-isogenic Mla line (resistant to powdery mildew). We compared changes in the expression of barley genes between the susceptible and resistant barley lines by RNA-Seq. Total RNA samples were taken 3 and 7 days post inoculation from powdery mildew inoculated and control leaves and the expression profiles of these RNA samples were examined. With the RNA-Seq method we obtained information about the expression of about 43,000 barley genes in both infected and control samples. The bioinformatical analysis of the large amount of expressional data is still running. For the validation of RNA-Seq data we carried out quantitative, real time qPCR investigations. During these investigations we measured the expression of several genes encoding RNase enzymes in order to compare the gene expression values with the results of earlier biochemical enzyme activity determinations (data not shown).

Investigation of apple - *Erwinia amylovora* interactions

To learn more about the role of sulfur-treatment in plant disease resistance, we decided to extend our experiments also to a plant-bacterium interaction. We selected the interaction of apple with the phytopathogenic bacterium *Erwinia amylovora* that causes the very damaging fire blight disease on apple and pear trees.

We carried out pre-treatments on apple flowers with aqueous solutions of sodium sulfide and sodium sulfite solutions containing Tween-20 as detergent, which were followed by inoculations with *Erwinia amylovora* under strictly controlled laboratory conditions. The chemical treatments had no effect on the visible disease symptoms.

Other activities

During the project we wrote three review papers about the significance of sulfur metabolism in plant - pathogen interactions (Gullner et al., 2018; Künstler et al., 2020a and 2020b).

Publications resulted from the project

Balogh E, Juhász C, Dankó T, Fodor J, Tóbiás I, Gullner G (2019) A zsírsav-deszaturáz gének aktiválódása paprika levelekben tobamovírus fertőzések hatására. Növényvédelem 80: 446-453.

Balogh E, Juhász C, Dankó T, Fodor J, Tóbiás I, Gullner G (2020) The expression of several pepper fatty acid desaturase genes is robustly activated in an incompatible pepper-tobamovirus interaction, but only weakly in a compatible interaction. Plant Physiol. Biochem. 148: 347-358.

Barna B, Máté G, Preuss J, Harrach BD, Gullner G, Manninger K, Fodor J (2022) Defence responses triggered by *Blumeria graminis* f. sp. *hordei* in non-host wheat genotypes results in a decrease in *Puccinia triticina* infection. J. Phytopathol. 170: 82-90.

Fodor J, Kámán-Tóth E, Dankó T, Schwarczinger I, Bozsó Z, Pogány M (2018) Description of the *Nicotiana benthamiana - Cercospora nicotianae* pathosystem. Phytopathology 108: 149-155.

Gullner G, Kőmíves T, Király L, Schröder P (2018) Glutathione S-transferase enzymes in plant-pathogen interactions. Front. Plant Sci. 9: 1836.

Kalapos B, Juhász C, Balogh E, Kocsy G, Tóbiás I, Gullner G (2021) Transcriptome profiling of pepper leaves by RNA-Seq during an incompatible and a compatible pepper-tobamovirus interaction. Sci. Rep. 11: 20680.

Kámán-Tóth E, Dankó T, Gullner G, Bozsó Z, Palkovics L, Pogány M (2019) Contribution of cell wall peroxidase- and NADPH oxidase-derived reactive oxygen species to *Alternaria brassicicola*-induced oxidative burst in *Arabidopsis*. Mol. Plant Pathol. 20: 485-499.

Künstler A, Király L, Kátay G, Enyedi AJ, Gullner G (2019) Glutathione can compensate for salicylic acid deficiency in tobacco to maintain resistance to *Tobacco mosaic virus*. Front. Plant Sci. 10: 1115.

Künstler A, Kátay G, Gullner G, Király L (2020) Artificial elevation of glutathione contents in salicylic acid-deficient tobacco (*Nicotiana tabacum* cv. Xanthi NahG) reduces susceptibility to the powdery mildew pathogen *Euoidium longipes*. Plant Biol. 22: 70-80.

Künstler A, Gullner G, Ádám AL, Kolozsváriné Nagy J, Király L (2020a) The versatile roles of sulfur-containing biomolecules in plant defense - a road to disease resistance. Plants (Basel) 9: 1705.

Künstler A, Gullner G, Schwarczinger I, Kolozsváriné Nagy J, Ádám A, Király L (2020b) A kén szerepe a növényi betegség rezisztenciában. Növényvédelem 81: 461-468.

Saja D, Janeczko A, Barna B, Skoczowski A, Dziurka M, Kornaś A, Gullner G (2020) Powdery mildew-induced hormonal and photosynthetic changes in barley near isogenic lines carrying various resistant genes. Int. J. Mol. Sci. 21: 4536.

References

Bloem E, Haneklaus S, Schnug E (2015) Milestones in plant sulfur research on sulfur-induced-resistance (SIR) in Europe. Front. Plant Sci. 5: 779.

Giraud E, Ivanova A, Gordon CS, Whelan J, Considine MJ (2012) Sulphur dioxide evokes a large scale reprogramming of the grape berry transcriptome associated with oxidative signalling and biotic defence responses. Plant Cell Environ. 35: 405-417.

Gruhlke MC, Slusarenko AJ (2012) The biology of reactive sulfur species (RSS). Plant Physiol. Biochem. 59: 98-107.

Kruse C, Jost R, Lipschis M, Kopp B, Hartmann M, Hell R (2007) Sulfur-enhanced defence: effects of sulfur metabolism, nitrogen supply, and pathogen lifestyle. Plant Biol. 9, 608-619.

Leustek T, Martin MN, Bick JA, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 141-165.

Rausch T, Wachter A (2005) Sulfur metabolism: a versatile platform for launching defence operations. Trends Plant Sci. 10, 503-509.

Shi H, Ye T, Han N, Bian H, Liu X, Chan Z (2015) Hydrogen sulfide regulates abiotic stress tolerance and biotic stress resistance in Arabidopsis. J. Integr. Plant Biol. 57: 628-640.

Tóbiás I, Fraser RSS, Gerwitz A (1989) The gene-for-gene relationship between *Capsicum annuum* L. and tobacco mosaic virus: effects on virus multiplication, ethylene synthesis and accumulation of pathogenesis-related proteins. Physiol. Mol. Plant Pathol. 35: 271-286.