GENETICA, BIOLOGIA MOLECULARĂ ŞI AMELIORAREA

IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED IN SUNFLOWER DURING INTERACTION WITH *PLASMOPARA HALSTEDII*

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Rezumat

Mana, cauzată de agentul patogen din clasa oomicetelor *Plasmopara halstedii*, este una dintre cele mai devastatoare boli ale florii-soarelui cultivate (*Helianthus annuus* L.). Cu toate acestea, interacţiunea între floarea-soarelui şi *P. halstedii* nu este bine studiată. Pentru identificarea genelor cu expresia diferenţiată în cazul reacţiei florii-

soarelui la *P. halstedii* a fost analizată activitatea transcipţională a genelor asociate cu răspunsul defensiv la cinci genotipuri de floarea-soarelui din RM, care au fost infectate natural cu mana. În total 14 secvenţe de gene şi opt EST-uri au fost luate în studiu. În baza clasificării după funcţie la plante model şi gradului înalt de omologie au fost atribuite categorii funcţionale secvenţelor studiate. Grupul cel mai mare a cuprins genele superoxid dismutazelor, catalazelor etc. Mai mult ca atât, în acest studiu au fost incluse genele implicate în transducerea semnalelor şi factori de transcripţie, expresaţi în timpul răspunsului defensiv la patogeni. Evidenţierea genelor cu expresie diferenţiată în răspunsul florii-soarelui la mana a relevat parţial mecanismul molecular asociat cu rezistenţa.

Cuvinte-cheie: *Helianthus annuus* L. – *Plasmopara halstedii* – răspunsul defensiv – genele cu expresia diferenţiată – PCR cantitativ.

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Introduction

Plants, unlike animals, have no immune system cells specialized for providing the defense mechanisms, but they are attacked by many pathogens, such as bacteria, oomicete, fungi, viruses, and nematodes which cause large losses of yield. During the evolution plants have developed complex mechanisms for recognizing and responding to the infection caused by a large number of pathogens [7].

Downy mildew of sunflower induced by *Plasmopara halstedii* F. Berl et de Toni is a major disease in all regions of the world, where sunflower is cultivated [9]. This pathogen spreads throughout the soil, air and seeds. Significant losses occure in more than 50% of infected plants in fields, and leading to yield losses of up to 80% [8]. *P. halstedii* belongs to the class Oomycetes with numerous representatives: saprophytic organisms, pathogens of plant, insects, fish, nematodes, vertebrates, and various classes of microorganisms [4]. Phytopathogenic oomycetes infect a wide range of host plants, including crops, weeds, ornamental plants and trees [6].

In order to stop the spread of the disease are used systemic fungicides with long-term properties, such as metalaxyl or similar compounds [13]. But the most effective method to prevent desease is growing of resistant hybrids [5, 14, 15]. The emergence of new pathogenic races questioning the successful cultivation of resistant hybrids in some regions [3,5] and there is a need to search and combine new sources of resistance with existing [14, 15].

As an alternative for specific resistance sources in sunflower can be quantitative, non-specific resistance, which is not dependent on host-pathogen interaction. These ideas were developed by Vear et al in 2006 and widely studied for three years. A significant level of partial resistance was shown for sunflower lines that do not possess effective *Pl* genes for known pathotypes. This type of resistance and pathogen virulence occurs regardless of heredity that is under additive control [12]. Quantitative resistance on sunflower has been studied by Serre et al in 2008 under greenhouse conditions, which highlighted the importance of plant age and interaction with the environment during the host response [10]. Another research have demonstrated the possibility of

obtaining of sunflower hybrids with partial resistance to downy mildew but with a high content of oil [2]. Recently, Tourvieille of Laboruhe et al suggested the importance of identifying forms with non-specific resistance to downy mildew and to include them in agricultural practice [11].

In this research, expression of some genes and their functional role in sunflower-downy mildew interaction were studied.

Materials and methods

Plant and pathogen material. Five sunflower genotypes (Drofa Rf, Drofa CMS, Drofa F_1 hybrid and two isogenic lines 393A and B) were grown in field during summer of 2011. Biological samples were collected in field from normal and naturally downy mildew infected plants with different degree of infection: systemic, medium, infected and non-infected leaves of weakly infected plants, control; frozen in liquid nitrogen and stored at – 80ºC.

Total RNA isolation and cDNA synthesis. Total RNA was isolated using TRI Reagent (*Ambion*), 0,6 µg of total RNA was treated with DNase (*Promega*) and first-strand cDNA was synthesized with RevertAid Reverse Transcriptase and Random hexamer and Oligo-dT18 primers *(Fermentas*) according to the manufacturer's instructions.

Gene expression study by qPCR. The primers were designed through Primer3Web v. 3.0.0. software for the sequences of interest from NCBI Nucleotide and EST databases. Gene expression was estimated using RT qPCR using Maxima SYBR Green/ ROX qPCR Master Mix (*Fermentas*) and Real-Time cycler DT-96 (*DNA technology*, Russia).

Sequence and bioinformatics analysis. Sequences were analysed using BLASTX [1] performed for sequences of model organism *Arabidopsis thaliana*. On the base of function, processes and cellular component established in model plant, such categories were assigned for investigated sequences.

Results and discussion

Several genes, known as modulators and key-elements of plant defense responses to biotic and abiotic stress were studied.

Functional categories of genes responsive to P. halstedii infection. For the 22 sequences was performed homology analysis with BLASTx tool in GenBank on the model organism *Arabidopsis thaliana*. Query coverage within selected group of genes varied from 31 % (*defensin*) to 100 % (*GST*). Identity ranged from 47 % (*GST*) to 87 % (*AOXIA*, *CATA1* and *CATA2*). For eight ESTs included in study were established similarities with following genes in Arabidopsis: L-ascorbate peroxidase, L-ascorbate peroxidase 1, alternative oxidase 1A, peroxiredoxin-2F, glutathione synthetase, transcription factor *TGA2*, transcription factor *TGA6* and single-stranded DNA-binding protein *WHY3* (Table 1).

The major part of investigated genes consist of enzymes with antioxidant functions, such as superoxide dismutases, catalases, peroxidases etc., which ensure efficient response to oxidative stress induced in cell during defense.

For investigated genes were attributed different functions, such as diverse types of enzymatic activities, protein binding, DNA binding and some of genes were not

attributed molecular function (category – unknown molecular function). The largest functional categories were: SOD activity (18 %), catalase activity (18 %), DNA binding (14 %), L-ascorbate peroxidase activity (9 %) and unknown molecular function (9 %). Other categories were 4-5 % each (Figure 1A).

Table 1. The list of genes and ESTs included in analysis

Gene/EST	GenBank accession no.	Accession no. of matching sequence	Matching sequence from database	E-value	Query coverage	Identity
Mn-superoxide dismutase II	DQ812552.2	NP_187703.1	Superoxide dismutase [Mn]	1e-133	73%	79%
Mn-superoxide dismutase I	DQ812551.2	NP_187703.1	Superoxide dismutase [Mn]	5e-135	71%	81%
Cu/Zn superoxide dismutase; (sod2 gene)	AJ786258.1	NP_172360.1	superoxide dismutase [Cu-Zn]	5e-80	62%	81%
Cu/Zn superoxide dismutase precursor; (sod1 gene)	AJ786257.1	NP_565666.1	copper/zinc superoxide dismutase 2	8e-93	75%	80%
Catalase	L28740.1	NP_195235.1	catalase 2	0.0	84%	87%
Catalase 3 (CATA3)	AF243518.1	NP_195235.1	catalase 2	0.0	79%	77%
Catalase 2 (CATA2)	AF243517.1	NP_195235.1	catalase 2	0.0	84%	87%
Catalase 4 (CATA4)	AF243519.1	NP_195235.1	catalase 2	0.0	78%	78%
Glutathione peroxidase	Y14429.1	NP_192897.2	glutathione peroxidase 6	2e-84	74%	70%
Phenylalanine ammonia lyase	Y12461.1	NP_181241.1	phenylalanine ammonia-lyase 1	0.0	89%	85%
NPR1 (NPR1)	AY667500.1	NP_193701.2	NPR1-like protein 4	3e-21	98%	65%
Defensin	AF364865.1	NP_173391.2	defensin-like protein 19	4e-18	31%	55%
Pathogenesis-related protein 5-1 (PR5-1)	AF364864.1	NP_192902.1	osmotin-like protein OSM34	6e-105	77%	69%
Glutathione S-transferase (GST)	AY667502.1	NP_180510.1	glutathione S-transferase tau 1	2e-14	100%	47%
QHJ20E24.yg.ab1	BU032190.1	NP_195226.1	L-ascorbate peroxidase	5e-123	91%	80%
DH0AC027-ZF05FM1	CD849992.1	NP_172267.1	L-ascorbate peroxidase 1	3e-25	50%	84%
CCFT3158.b1_K21.ab1	GE502151.1	NP_188876.1	alternative oxidase 1A	2e-140	87%	87%
CCFU1199.b1_M11.ab1	GE515985	NP_566268.1	peroxiredoxin-2F	7e-95	82%	74%
DH0ANA3ZD01-FM1	CX946030.1	CAA58318.1	glutathione synthetase	6e-72	60%	72%
CCFU2042.b1_D07.ab1	GE516848.1	NP_196312.1	transcription factor TGA2	2e-98	82%	75%
CCFT8591.b1_N11.ab1	GE512673.1	NP_566415.3	transcription factor TGA6	2e-92	95%	74%
CCFS537.b1_B15.ab1	GE491965.1	NP_001077872.1	single-stranded DNA-binding protein WHY3	2e-104	71%	73%

*** Actin (reference gene) AF282624.1**

Investigated genes and ESTs showed in previous studies made on Arabidopsis implication in different processes, such as response to metal ions; response to biotic stress, defence response; response to abiotic stress, caused by different factors – salt, heat, cold, ozone, etc.; response to starvation of different elements; response to oxidative stress; toxin catabolic process; glutathione biosynthetic process; transcription regulation and SAR. The largest category – 28 % included sequences involved in responses to abiotic stresses induced in various conditions. 22 % were accounted for "response to oxidative stress". Another large categories were "response to metal ions", such as zinc, copper, iron and cadmium and "response to biotic stress, defense response" – 15 % each (Figure 1B).

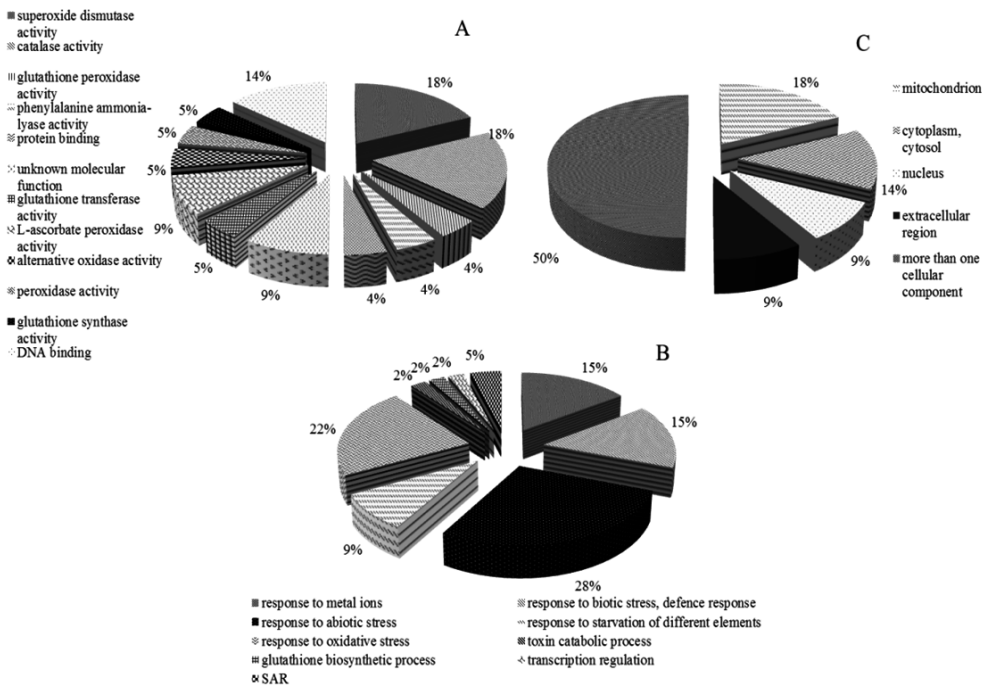

Figure 1. Functional categories (A), molecular process (B) and cell localization (C) of studied genes.

Investigations regarding cell localization of studied transcripts revealed that the major part (50 %) of these are localized in more than one cell compartment, 18 % in mitochondria, 14 % in cytosol or cytoplasm, 9 % in nucleus and 9 % in extracellular region (Figure 1C). In extracellular region were located transcripts than have defense functions against pathogen attacks – *PR5* and *defensin*.

Thus, using data from NCBI databases and available bioinformatics tools was performed a brief analysis regarding functions, processes and cell localization of sequences from gene set selected for expression analysis. It revealed that most of these sequences are implicated in metabolic pathways, especially in response to oxidative stress and have enzymatic activity function.

Gene expression studies during sunflower-downy mildew interaction. Gene expression studies performed for cDNA samples collected from systemic, medium and

weakly infected plants revealed differential expression of investigated genes in function of infection degree (Figure 2). There are genes which are up-regulated in weakly infected plants and non-infected tissues of these (*SOD* genes, *CATA1*, *GSS*, *Why1*, *defensin*, *APX1*) and in systemic and medium infected plants (*CuZnSOD II*, *CATA3*, *CATA4*, *GST*, *NPR1*, *TGA5*, *PAL*) or in all cases (*CuZnSOD I*, *PR5*). These genes are predominantly involved in superoxide metabolism, SAR and defense reponses.

The increase in expression of the gene encoding the transcription factor *Why1*, in the weakly infected plants suggested that the establishment SAR in infected and non-infected parts these plants is independent from *NPR1*.

Differential down-regulation was demonstrated for eight genes in systemic and medium infected plants (*MnSODs*, *CuZnSOD I*, *CATA1*, *CATA2*, *Why1*, *defensin* in isogenic lines, *APX1*), four genes in weakly infected plants *(PRXIIF*, *GPX*, *TGA5*, *PAL*) and two genes (*AOXIA* and *APX3*) suppressed in both cases, appeared to be predominantly involved in superoxide and peroxide metabolism.

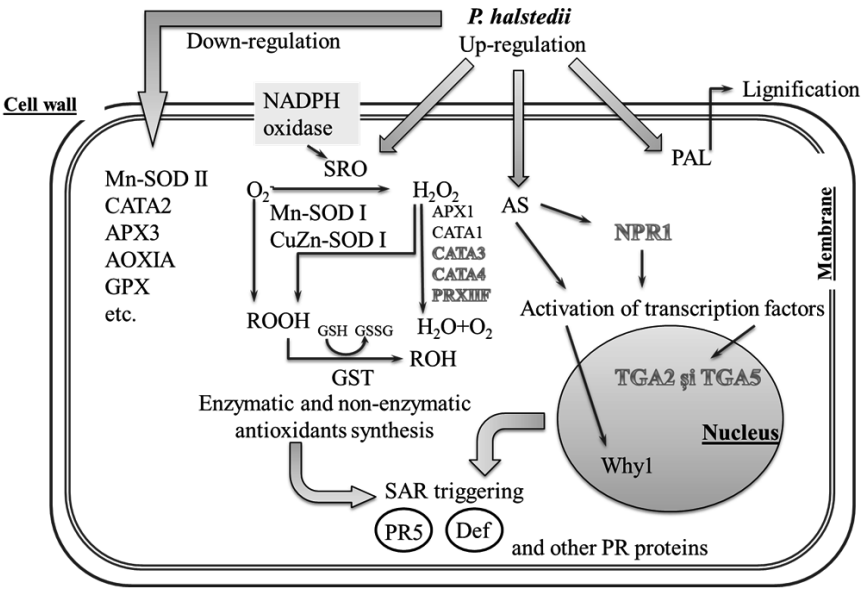

Figure 2. Hypothetic model which elucidates mechanisms associated with sunflower resistance to downy mildew.

Significant changes in the pattern of gene expression of antioxidant enzymes were detected: up-regulated expression of mitochondrial *Mn-SOD I*, chloroplastic *CuZn-SOD I, CATA1*, *GST*, *APX1* in weakly infected plants, and *CATA3, CATA4, PRXIIF* in systemically and medium infected or down-regulated expression of *Mn-SOD II, CATA2, APX3, GPX.* Such modifications ensure physiological process of efficient detoxification and scavenging of ROS, using minimal energy.

In conclusion, it could be mentioned that as a result of sunflower infection with downy mildew it is activated a series of general defense mechanisms, which differ significantly in terms of resistance potential of host plant (Figure 2). Elucidating the genetic aspects of resistance as well as the fundamental defense mechanisms will contribute significantly to facilitating the process of obtaining resistant hybrids.

Conclusions

Genes selected for analysis have functions such as diverse types of enzymatic activities, especially superoxide and peroxide dismutation; protein binding; DNA binding and other. Some of these genes are up-regulated during infection (*CuZnSOD II, PR5*), other were down-regulated (*AOXIA, APX3*). It was observed relationship between infection degree (weakly, medium and systemic infected plants) and expression profile of studied genes.

Expression and functional analysis of these genes helped us to understand some aspects of the molecular mechanism of resistant defense response in sunflower against *P. halstedii*.

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