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Proteomic Analysis of Salicylate-Induced Proteins of Pea (*Pisum sativum* L.) Leaves

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Abstract—The effect of 50 μM salicylic acid on soluble proteins of pea (*Pisum sativum* L.) leaves was studied by proteomic analysis. Thirty-two salicylate-induced proteins were found, and 13 of these were identified using MALDI TOF MS. Salicylate-induced increased content was shown for the first time for the family 18 glycoside hydrolase, α-amylase, 33 kDa protein of photosystem II, lipid-desaturase-like protein, and glutamine amidotransferase. Increased content of protective proteins of direct antipathogenic action such as chitinase and β-1,3-glucanases was also noted.

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The stress phytohormone salicylic acid (SA) is one of the key endogenous regulators of plant resistance and immunity [1-4]. Rapid significant increase in SA content in plant tissues ("salicylate burst") in response to a pathogen or elicitor effects have been found [5].

Treatment of plants with exogenous SA stimulated synthesis of protective proteins, absent from control plants, and simultaneously inhibited development of pathogenic microorganisms and increased the resistance of plant cells to them. This effect is due to the ability of SA to act as a signal molecule "launching" signal metabolic chains and also as a mediator in superoxide synthase and NO-synthase signal systems [6]. The SA-induced change in their activity should inevitably result in reprogramming of gene expression and protein synthesis. In recent years, proteomes of various plant organs (leaves, stems, roots) and effects on them of pathogens, elicitors, phytohormones, and signal system mediators have been intensively studied [7-10]. Many new SA-induced proteins have been identified using proteomic analysis. Thus, we have detected for the first time [11, 12] the SA-induced increase in the content in pea roots of quite a number of auxiliary proteins incorporated in heteromeric supramolecular complexes involved in DNA transcription and repair, protein translation, degradation, and refolding, as well as in activation of the cell signal system mediators. Since proteomes of various plant organs differ significantly, it is important to study on the same plants [11, 12] the effect of SA on the leaf proteomes.

MATERIALS AND METHODS

Reagents. The following reagents were used throughout this work: acrylamide, Chaps, N,N′-methylene-bis-acrylamide, thiourea, Triton X-100, cocktail of protease inhibitors, and SA from Sigma (USA); dithiothreitol (DTT), iodoacetamide, IPG strips, Bio-Lyte pH 3-10 (4-7), mineral oil, urea, and markers for two-dimensional (2-D) electrophoresis from Bio-Rad (USA); and modified trypsin (Promega, USA).

Plant material. Pea (*Pisum sativum* L., Tan variety) seedlings were grown in tap water at 25°C illuminated by luminescent lamps with illumination intensity of 10 klx with 16 h light period. Roots of 8-day-old pea seedlings were placed into 50 μM SA solution. Seedlings grown in water served as control. In 5 days leaves of 1 g samples were fixed in liquid nitrogen. Our earlier experiments have shown that at shorter exposures (1-3 days) changes

Abbreviations: Bio-Lyte (pH 3-10), ampholytes; Chaps, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (nonionic detergent); 2-D electrophoresis, two-dimensional electrophoresis; IEF, isoelectric focusing; NPR1, protein that does not cause expression of the pathogen-induced protein PR1 (non-expressor of PR1); SA, salicylic acid; TCA, trichloroacetic acid.

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in the protein spectrum and content already begin, but they do not reach the levels detected after 5 days of root exposure in SA solution. A similar conclusion about optimal 4-7-day-long exposure was drawn by other authors who studied by one-dimensional electrophoresis the effect of SA on formation of PR proteins in sunflower leaves [13]. Three independent experiments were carried out.

Extraction of soluble proteins. Soluble proteins were extracted from leaves using 0.025 M Na-acetate buffer, pH 5.2, in the presence of 100 mM DTT and 1% protease inhibitor cocktail at 4°C. Specimens were centrifuged for 10 min at 14,000*g*, and then an equal volume of ice-cold 20% (w/v) trichloroacetic acid (TCA) solution in acetone was added to the supernatant containing soluble proteins. The proteins were kept for 1 h at −20°C. The precipitated protein was washed three times with cooled acetone in the presence of 50 mM DTT. The precipitate was dissolved in IEF buffer containing 6 M urea, 2 M thiourea, 2% Chaps, 2% Triton X-100, 50 mM DTT, and 0.5% Bio-Lyte, pH 3-10.

Quantitative protein determination. Ten microliters of protein solution was adjusted with water to 150 μl and precipitated by equal volume of cooled 20% TCA. The precipitate was dissolved in 0.1 M NaOH. Protein concentration was determined by the Lowry method [14] with BSA as standard.

Two-dimensional electrophoresis. 2-D electrophoresis was carried out on Protean IEF Cell and Protean II xi 2D Cell devices (BioRad) using 17 cm gel strips with immobilized pH 4-7 and 3-10 linear gradients. Samples for application on the strips contained equal amount of protein (400 μg). Protein samples were introduced into the buffer for isoelectric focusing containing 6 M urea, 2 M thiourea, 4% Chaps, 50 mM DTT, 0.5% Bio-Lyte (pH 3-10), and one drop of bromophenol blue. Strips were rehydrated at 50 V for 12 h and isoelectrofocused for 10-12 h at 20°C. First, the sample was desalted at 250 V for 15 min, and then the voltage was increased linearly to 10,000 V during 5 h, after which it was isoelectrofocused for 5-6 h to achieve a total of 60,000 Vh. The beginning of protein separation in the second direction was preceded by equilibration of the strips for 15 min in the first buffer (0.125 M Tris-HCl, 2% SDS, 2% DTT, 30% (w/v) glycerol, 6 M urea, and traces of bromophenol blue), and then strips were kept for 15 min in the second equilibrating buffer containing 2.5% iodoacetamide instead of DTT. Proteins were separated by molecular mass using 12.5% SDS-PAGE at 40 mA per gel. Gel plates were stained with Coomassie G-250, which enabled quantitative estimation of protein content. Gels were scanned on an EPSON 4990 Photo scanner (Selico EPSON Corporation, Japan). The gel scans were analyzed using the Phoretix 2D v 2004 program (Nonlinear Dynamics). Three repetitions of control and experimental variants were analyzed.

Tryptic protein hydrolysis. Gel pieces of 1 × 1 mm were washed with 40% acetonitrile solution in 0.1 M NH_4HCO_3 for 30 min at 37°C to remove the dye and then dried, and then they were hydrolyzed using modified trypsin at concentration 15 μg/ml. The samples were hydrolyzed for 2 h at 56°C, and the reaction was stopped by addition of 0.5% trifluoroacetic acid in 10% acetonitrile. MALDI mass spectra were recorded in supragel solutions.

Protein identification using mass spectrometry. Mass spectra were obtained using a Bruker (Germany) Ultraflex II tandem MALDI-TOF-TOF mass-spectrophotometer equipped with UV laser (Nd). Mass spectra were obtained in the positive ion regime using a reflectron. The precision of measured fragment mass values was 1 Da. Proteins were identified using the Mascot program (www.matrixscience.com). The samples were identified by "peptide fingerprint" search in the NCBI (National Center for Biotechnology Information) database (Green plants) with indicated accuracy with regard to the possible oxidation of methionine by the air oxygen and possible modification cysteine by acrylamide. Proteins that escaped determination by peptide fingerprint were determined using MS/MS peptide fragmentation. The MS/MS and MS + MS/MS results were searched using the Biotools 3.0 software (Bruker Daltonics, Germany). Mass spectra (MS and MS/MS) were determined and proteins were identified in the Institute of Physicochemical Medicine of the Russian Federation Ministry of Health.

RESULTS AND DISCUSSION

SA-induced changes in protein content. The 5-day-long maintenance of pea seedling roots on SA solution caused only slight changes in total content of the leave soluble proteins (in control plants 17.5 ± 0.1, and in experimental plants 18.8 ± 0.8 mg/g wet weight). This suggests that in our experiments the comparison of the content of individual proteins by proteomic analysis does not require correction for the change in the total protein content in the leaves.

A large share of the soluble proteins in leaves (30-50%) is known to be proteins of the Calvin cycle [15, 16]. In our experiments it was found during protein extraction with 0.025 M Tris-HCl, pH 8.0, and their following separation by 2-D electrophoresis that the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RBPC/O) as well as other enzymes of the Calvin cycle formed the highest content of soluble proteins (Fig. 1). As a result, minor proteins are inevitably not visualized after protein identification on 2-D electrophoregrams. Since RBPC/O is not extracted in acidic buffer (pH 4.6-5.2) (unlike most pathogen- and elicitor-induced proteins), we used a variant with protein extraction in sodium acetate buffer, pH 5.2 [13].

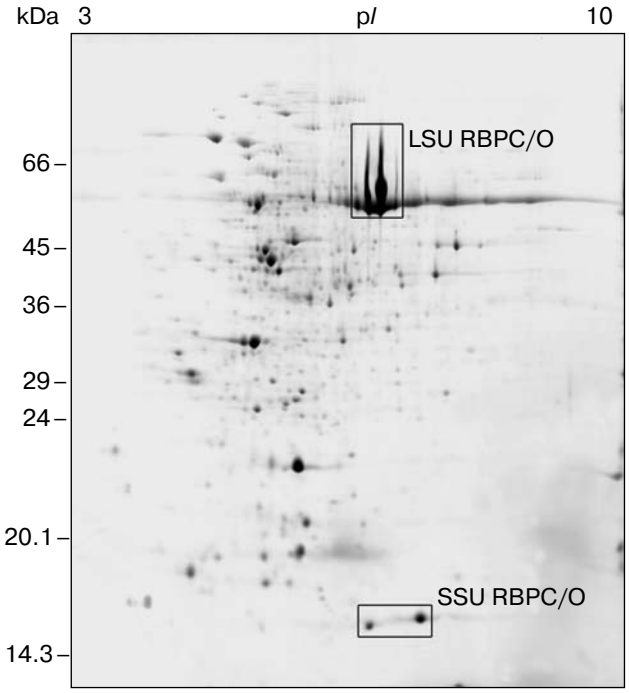


Fig. 1. 2-D-Electrophoregram of pea leaf soluble proteins extracted with 0.025 M Tris-HCl, pH 8.0. LSU, large subunit; SSU, small subunit of RBPC/O.

In the case of separation on strips at pH 4-7 (Figs. 2 (a and b) and 3), over 600 protein spots were revealed using the Phoretix 2D v 2004 computer program (Nonlinear Dynamics). SA induced changes in the content of 28 proteins, seven of which (spots 3, 4, 7, 8, 9, 13, 26) were not found in the control. Protein 1, characteristic of control, was absent from the SA-treated seedling leaves. Rectangles in Fig. 2 designate A-C regions in which the most significant changes in protein content were observed.

Upon separation on strips at pH 3-10, in the alkaline region (rectangle D), the SA-induced formation of three proteins (29-31), absent from control, was found along with significant increase in the content of protein 32 (Figs. 2c and 3).

Identification of SA-induced proteins. We identified 13 of 32 of the above mentioned SA-induced proteins (table). The search by "peptide fingerprint" revealed four proteins. The other proteins were identified using further MS/MS peptide fragmentation.

The SA-induction of many proteins belonging to different glycoside hydrolase families attracts attention. The increase in content of α-amylase (protein 2) belonging to glycoside hydrolase family 13 as well as of glycoside hydrolase of family 18 (protein 17) was found for the first time.

α-Amylase catalyzes the first reaction in the metabolic chain of starch degradation, and the increase in its content is able to provide for glucose supply for biosynthesis of different carbohydrates and for respiration. It is known that exogenous SA is able to intensify respiration in plant tissues.

Among family 18 glycoside hydrolases there are class III and IV classical chitinases, hydrolyzing β-1,4-N-acetyl-D-glucosamine bonds in chitin molecules of cell walls of pathogenic fungi, and so-called inactivated chitinases, inhibitors of xylanases, enzymes produced by pathogenic fungi [17] for degradation of hemicelluloses in plant cell walls. Family 18 also includes chitinases exhibiting lysozyme activity and able to hydrolyze β-1,4-bonds between N-acetylglucosamine residues and residues of acetylmuramic acid in murein-peptidoglycan incorporated in bacterial cell walls [18]. The SA-induced enhancement of synthesis of chitinase (protein 32), belonging to glycosyl hydrolase family 19, as well as of isoforms of acidic and alkaline β-1,3-glucanases (proteins 3, 13, 14, 30, 31) of glycoside hydrolase family 17, previously found by other authors [16, 19], was also detected. The simultaneous SA-induced increase in the chitinase and β-1,3-glucanase content can be highly expedient, because their combined action causes synergistic inhibition of development of pathogenic fungi [20].

It is interesting that we did not find in pea roots the SA-induced increase in the chitinase and β-1,3-glucanase content [11, 12], while in alfalfa plants constitutive chitinases were found in roots, but not in leaves [21]. It is possible that in the legumes plant roots chitinases are present mainly due to constitutive synthesis and in leaves their presence is due to inductive (in our case SA-induced) synthesis. Data indicating that the plant roots contain almost double, compared to leaves, amount of constitutive proteins responsible for immunity and resistance are consistent with this conclusion [8, 9].

We have shown for the first time the SA-induced increase in leaves in plastid protein content, the lipid-desaturase-like protein and 33 kDa protein of photosystem II.

The SA-induced increase in the content of lipid-desaturase like protein (protein 9), mainly localized in the leaf chloroplasts, attracts special attention because desaturation of saturated fatty acids is necessary for synthesis of polyene fatty acids serving as a source of hydroperoxide derivatives synthesized during lipoxygenase reactions and involved in the lipoxygenase signal cascade. Many oxylipins formed in this case are stress phytohormones, signal compounds, and volatile and nonvolatile antibiotics [22, 23]. The enhancement of formation of transcripts of fatty acid desaturase upon plant infection by a pathogen was noted earlier [24]. The desaturase-like protein can also be involved in biosynthesis of cutin and suberin [25], which are protective barriers on the way of pathogen penetration into plants.

The 33-kDa protein of photosystem II (protein 22) is the magnesium-stabilizing protein playing an important

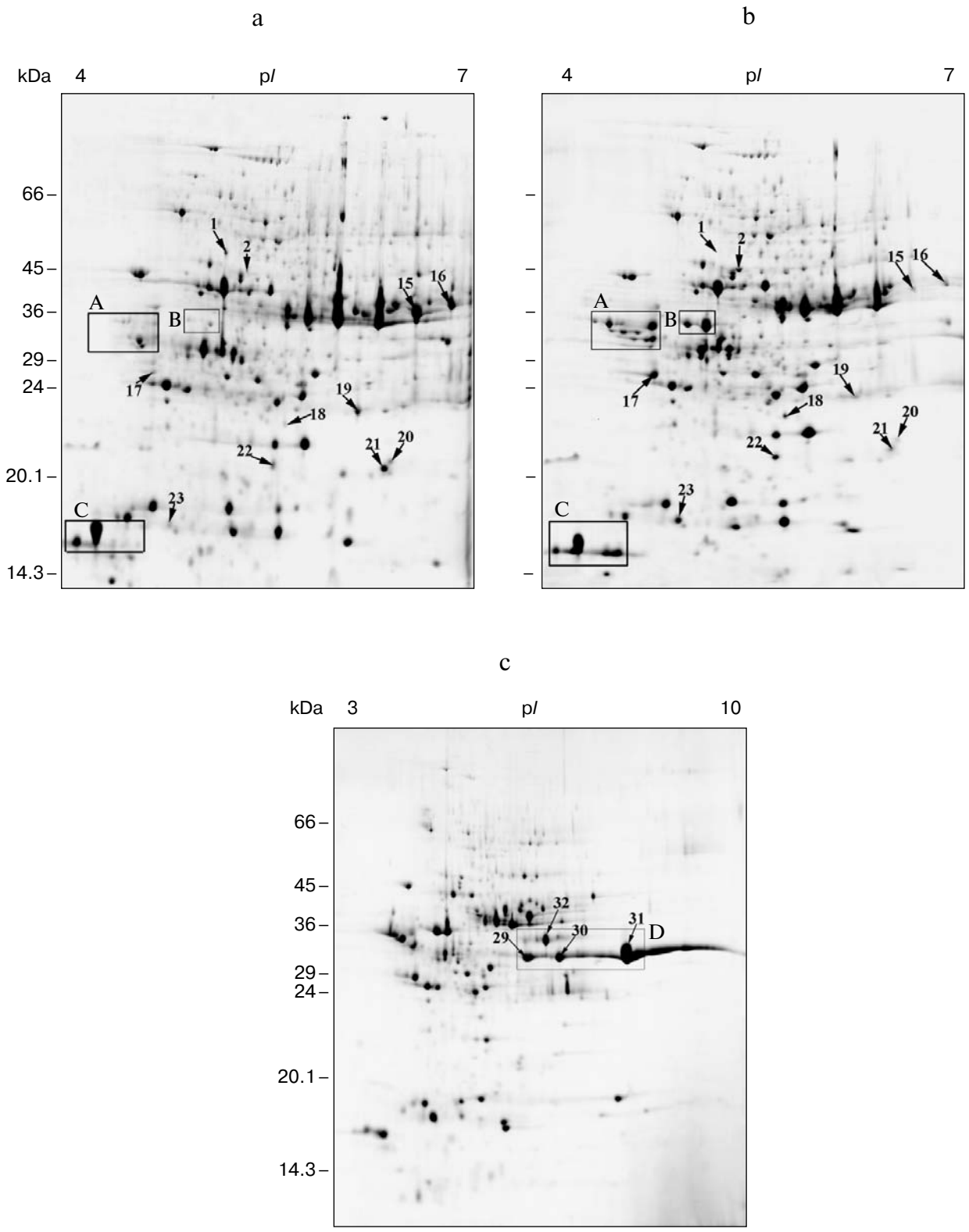


Fig. 2. 2-D electrophoregrams of soluble proteins of pea leaves extracted with 0.025 M Na-acetate buffer, pH 5.2. a) Control; b, c) 50 μM salicylic acid-treated. 2-D electrophoresis was performed on strips at pH 4-7 (a, b) or at pH 3-10 (c). Gels were stained with Coomassie G-250. Molecular mass values of protein markers are shown to the left. A-D, distinguished gel regions (numbers designate SA-induced proteins).

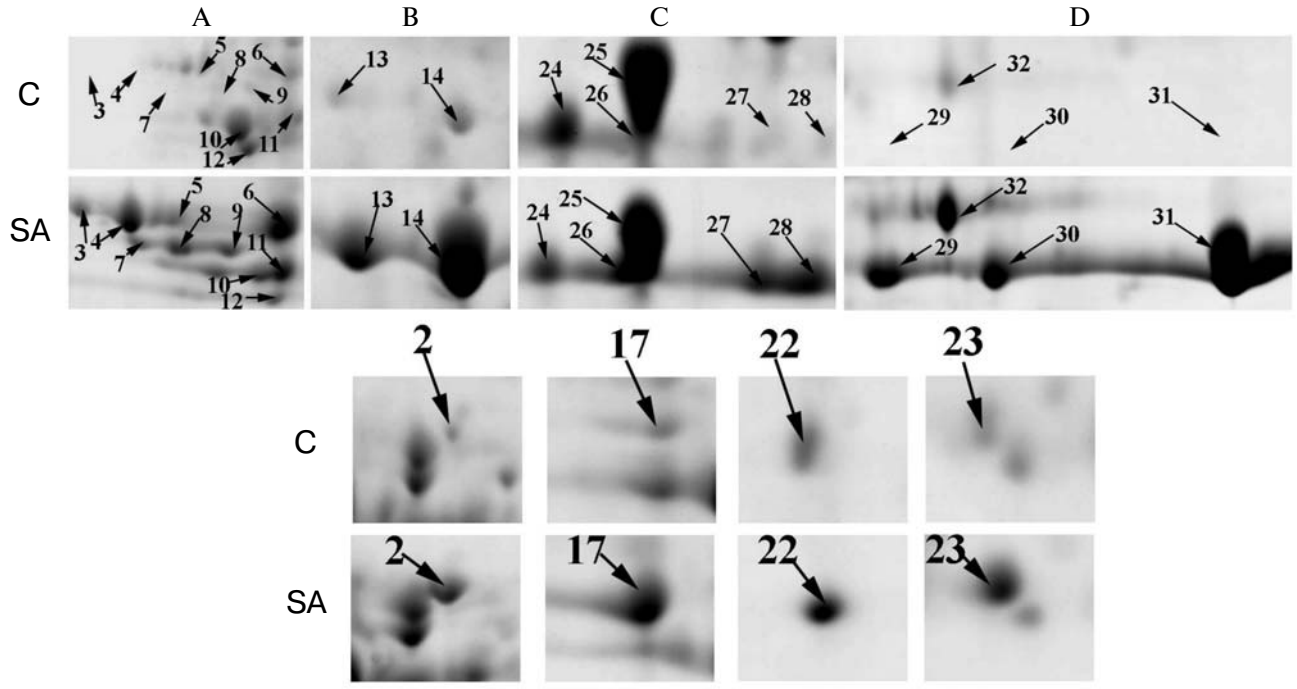


Fig. 3. Gel fragments of 2-D electrophoresis. C, control; SA, 50 μM salicylic acid-treated.

role in functioning of the O_2-evolving chlorophyll–protein complex [26]. The increase in the content of the 33-kDa protein of photosystem II can provide for higher resistance of this "hot section" of the photosynthesizing cell.

We have also shown for the first time the SA-induced increase in the content in leaves of glutamine amidotransferase class I domain-containing protein (protein 29), potentially enhancing the use of glutamine for synthesis of purine and cytosine nucleosides followed by generation of nucleoside triphosphates for DNA and RNA syntheses.

The induction by SA of the disease resistance response protein (protein 23), whose synthesis was significantly enhanced in response to various pathogens, was found [27].

The content of nine soluble proteins decreased, and we have identified among them protein 15, the precursor of malate dehydrogenase. Different malate dehydrogenase isoforms are localized in chloroplasts, mitochondria, and cytosol. They are involved in reducing equivalent "shuttle" transfer between chloroplasts, cytosol, and mitochondria, in the citric acid cycle in mitochondria, in β-oxidation of fatty acids, and in many other redox reactions. Physiological significance of the decrease in the content in leaves of SA-dependent malate dehydrogenase is not been explained because its localization has not been identified.

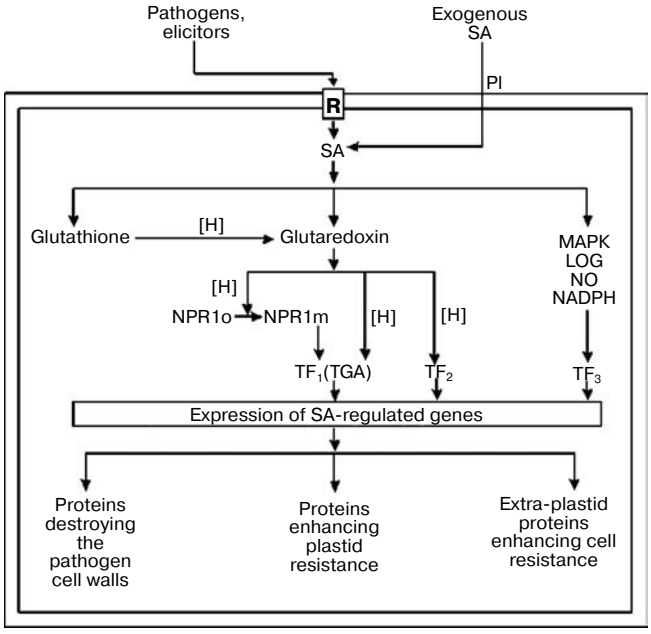
Possible mechanisms of the SA effect on proteomes. The question concerning signal mechanisms causing changes in plant proteomes in response to SA is still not quite clear. There are data on the existence of SA-activated MAP kinases (SIPK) [28, 29] in the MAPK signal system, on SA-activation of lipoxygenase [30], NADPH-oxidase [31], and NO-synthase [32, 33] signal systems.

Recently new and interesting data have been obtained concerning possible reprogramming mechanisms of protein synthesis regulation in plants. A special role in this process of oxidoreductase changes of some participants of SA-signaling was detected. Studying the *Arabidopsis* mutant not expressing the pathogen-induced protective protein PR1 [34] made it possible to identify a special role of redox transformations of NPR1 protein in SA-regulation of protein synthesis. It was shown later that SA enhances NPR1 gene expression [35, 36], and conversion of this protein from oligomeric to monomeric form (due to disulfide bond reduction). This is a necessary condition of its transport from the cytosol into the nucleus and its interaction there with its "own" transcription factors of TGA family, resulting in binding of these factors with promoter regions of the PR gene array, regulation of their expression, synthesis of PR proteins, and finally induction of the systemic resistance of plants to pathogens. Glutathione [37] and the protein glutaredoxin [38], the contents of which increase upon SA treatment of plants (Scheme), take part in NPR1 activation as redox mediators. It is important to note that the above mentioned redox mediators also activate transcription factor TGA by reducing its conservative disulfide group, which is a necessary condition for its interaction with NPR1.

Identified SA-induced proteins of pea leaves

Spot number in gel[1]	Protein number in NCBI database[2]	Identified proteins[3]	MW/p*I*[4]	MW/p*I*[5]	Number of peptides/ peptide fragment[6]	Sequence coverage, %[7]	Score[8]	SA/C protein ratio[9]
2	gi|92887505	α-amylase, catalytic region (*M. truncatula*)	45.0/5.4	46.7/5.17	10	19	73	+5.8
3	gi|2921317	β-1,3-glucanase 3 (*Glycine max*)	34.1/4.34	26.1/4.27	IYYPDEE-ALQALR	5	98	E
9	gi|1161568	lipid desaturase-like protein (*Lycopersicon esculentum*)	33.1/4.66	38.7/8.87	MGGGG-NMSAM-TSKNEQK	10	84	E
13	gi|2921317	β-1,3-glucanase 3 (*Glycine max*)	33.7/5.01	26.1/4.27	(QEVVD-LYK) IYY-PDEEAL-QALR	24	74	E
14	gi|2921317	–"–	33.1/5.13	26.1/4.27	IYYPDEE-ALQALR	24	159	+15.3
15	gi|2827080	malate dehydrogenase precursor (*M. truncatula*)	40.2/6.55	35.8/6.4	10	29	84	–24.6
17	gi|92883858	glycoside hydrolase, family 18 (*M. truncatula*)	26.8/4.8	31.4/4.88	YGGVMI-WDRFN-DGQSG-YSNAIK	7	229	+7.3
22	gi|224916	33 kDa protein of photosystem II (*P. sativum*)	20.8/5.59	26.6/6.3	6	26	73	+1.9
23	gi|436313	disease resistance response protein (*P. sativum*)	17.0/4.95	16.7/4.94	10	75	144	+2.7
29	gi|50252075	glutamine amidotransferase class-I domain-containing protein (*Oryza sativa*)	33.2/6.5	32.3/6.02	VIDGEF-PAAEEV-GR	26	94	E
30	gi|82949446	β-1,3-glucanase (*Sesbania rostrata*)	33.2/7.25	36.5/9.03	HFGLFR-PDK	6	62	E
31	gi|82949446	–"–	33.2/8.5	36.5/9.03	HFGLFR-PDKSPK	5	112	E
32	gi|20687	chitinase (*P. sativum*)	35.5/7.1	34.5/7.83	GPIQLT-HNYNY-GLAGQ-AIK	26	167	+62.3

[1] Spot number in 2-D electrophoregram.
[2] Protein identification number in NCBI database.
[3] Identified protein of *P. sativum* or homologous protein of different organisms.
[4] MW/p*I*, experimental MW (protein molecular mass, kDa) and p*I*.
[5] MW/p*I*, theoretical protein molecular mass (kDa) and p*I* from database.
[6] Number of peptides used for protein identification or peptide fragment used for MS/MS studies.
[7] Coincidences in amino acid sequence.
[8] Reliability of protein score in NCBI database (plant sub-section) using the Mascot program where the windowing limit was 67 ($p < 0.05$).
[9] Ratio of protein content corresponds to mean of values measured in three independent experiments; "+" and "–", the increase and decrease in protein content; E, emergence of a protein absent from control.



Simplified scheme of SA regulation of synthesis of protective proteins. MAPK, MAP kinase; LOG, lipoxygenase; NO, NO synthase; NADPH, NADPH oxidase signal systems; NPR1o, oligomeric NPR1 protein; NPR1m, monomeric NPR1 protein; TF1-TF3, transcription regulation factors TF1-TF3; Pl, plasmalemma; R, receptor

Study of *Arabidopsis* mutants revealed the SA-induced NPR1-independent expression of some PR genes. It is supposed [38] that glutaredoxin is able to activate some factors of transcription regulation directly, without participation of the auxiliary protein NPR1. The possibility of SA-induced activation of transcription factors using still unknown regulatory proteins, different from NPR1, is also a possibility (Scheme).

Most likely, the contribution of various donors to glutathione and glutaredoxin reduction can change depending on the biological material investigated and the experimental conditions. In leaves, chloroplasts (extent of light-dependent reducibility of their plastoquinone pool and thioredoxin system) might play an important role in the shift of the redox status of chlorophyll-containing cells toward the reducing side [39]. The extent of reduction of participants of redox pairs (oxidized/reduced mediators) can also be strongly dependent on intensive production of reactive oxygen species in chloroplasts observed under the influence of different stress factors on plants. In the cytoplasm, the oxidative pentosophosphate cycle and triose phosphate dehydrogenase reaction of glycolysis can be a source of reducing equivalents.

It should be noted in conclusion that most of the SA-induced proteins identified by us in leaves might be involved in plant protective functions. In this case some of them might destroy cell walls of pathogenic microorganisms (chitinase, family 18 glycoside hydrolase, β-1,3-glucanases), and others can enhance the resistance of plastids (33-kDa protein of photosystem II) and cells as a whole (Scheme).

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