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BRIEF COMMUNICATION

Two-dimensional electrophoretic analysis of salicylic acid-induced changes in polypeptide pattern of barley leaves

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Abstract

The salicylic acid-induced changes in the polypeptide patterns of barley (*Hordeum vulgare* L.) leaves have been analysed using two-dimensional gel electrophoresis. An optimized 2-D PAGE protocol was used and gave reproducible 2-D gels from leaf crude protein extracts with a high number of detected polypeptides. When applied for 24 h SA affected the expression of a number of soluble proteins. Most of them appeared to be down-regulated. Although no abundant expression of specific proteins was observed, we detected three polypeptides that were present only in SA-treated leaves.

Additional key words: 2-D PAGE, *Hordeum vulgare*, leaf proteins.

Salicylic acid (SA) is an ubiquitous plant phenolic that controls plant growth and development (Schettel and Balke 1983), photosynthesis and transpiration rates (Pancheva *et al.* 1996), ion uptake and transport (Harper and Balke 1981), and induces changes in leaf anatomy and chloroplast ultrastructure (Uzunova and Popova 2000, Stoyanova and Uzunova 2001), and inhibition of ethylene synthesis (Leslie and Romani 1986). SA has been recognised as an endogenous regulatory signal in plants mediating plant defence against pathogens (Malamy *et al.* 1990, Durner *et al.* 1997). There is also evidence that SA can alter the antioxidant capacity and to induce thermotolerance in plants (Chen *et al.* 1997, Dat *et al.* 1998). Exogenous application of SA or its derivate acetyl-SA induced accumulation of pathogenesis-related (PR) proteins and an increased resistance to viruses and fungi (Ward *et al.* 1991, Enyedi *et al.* 1992). However, the function of most SA-induced proteins is largely unknown. Maslenkova and Toncheva (1998) demonstrated that SA altered polypeptide composition of thylakoid membranes and led to liberation of the 33 kD peripheral polypeptide. It is now apparent that changes in SA may play a role not only in pathogenesis but also in UV, ozone, and heat stress (Yalpini *et. al.* 1994, Sharma *et al.* 1996). The fact that PR proteins may appear in all of these stresses implies some cross-talk between their signalling pathways (Margis-Pinheiro *et al.* 1993).

Recently we have shown that long-term (7-d) treatment of barley plants with SA caused a decrease in the content of total soluble protein and of Rubisco in particular (Pancheva and Popova 1998). In short-term (upto 24 h) treated plants, however, changes in leaf proteins were not observed. It was suggested that SA had induced minor changes in protein expression.

The aim of this study was to apply a high resolution 2-D electrophoresis to analyze changes in polypeptide composition of barley leaves in response to short-term treatment with SA.

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Abbreviations: EDTA - ethylendiamine tetraacetic acid; IEF - isoelectric focusing; PAGE - polyacrylamide gel electrophoresis; pI - isoelectric point; PMSF - phenylmethyl sulfonyl fluoride; PP - polypeptide; SA - salicylic acid; SDS - sodium dodecylsulfate; 2-D - two-dimensional.

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Barley plants (*Hordeum vulgare* L. cv. Alfa) were grown in soil as described previously (Metodiev 1998) at photosynthetic photon flux density of 150 μmol m^{-2} s^{-1} and 12-h photoperiod. Growth temperature was 27 °C during the day and 20 °C during the night, and the relative air humidity was about 60 %.

Seven-day-old seedlings were cut under water and placed in glass flasks each containing 10 cm^3 of either 500 μM SA (pH 4.6 - 4.8) or distilled water as a control. The seedlings were left for a period of 24 h in light before extraction of proteins.

Fresh first-leaf tissue (200 mg) was homogenised in a mortar and pestle in 0.4 cm^3 extraction buffer which contained 50 mM HEPES-NaOH, 2 mM EDTA, 10 % (v/v) glycerol, 2 mM PMSF, 0.3 % (v/v) β-mercapto-ethanol (pH 7.5). The homogenate was centrifuged at 15 000 *g* for 15 min. Protein content of the supernatant was determined according to Bradford (1976).

One-dimensional SDS-PAGE was performed according to Laemmli (1970). An aliquot of the leaf extract was mixed with 4 × SDS-sample buffer (3:1), heated for 3 min in boiling water and 20 μg of proteins loaded per lane. Separation was conducted in 10 % polyacrylamide gel at constant current 20 mA per gel using mini-dual vertical gel electrophoresis apparatus (*Sigma-Aldrich*, Taufkirchen, Germany). After electrophoresis gels were fixed for 30 min in methanol:acetic acid:H_2O (40:10:50, v/v/v) and stained overnight with colloidal Coomassie stain. The stain contained 10 % (m/v) ammonium sulphate, 2 % (v/v) phosphoric acid and 0.1 % (m/v) Coomassie Brilliant Blue G250. Stained gels were then transferred to distilled water without any other destaining steps.

Two-dimensional electrophoresis was carried out following the original method of O'Farrell (1975) with some modifications. Sample solubilization buffer contained 2 % (m/v) CHAPS, 9.5 M urea and 2 % (v/v) β-mercaptoethanol. An aliquot of the leaf extract was mixed with solubilization buffer (1:3) and 20 μg of proteins loaded per gel. Isoelectric focusing (IEF) was performed in tubes (1.5 mm i.d. and 75 mm length). Gel solution contained 8 M urea, 2 % (m/v) *CHAPS*, 1.6 % (v/v) *Pharmalyte* (pH 5 - 8), and 0.4 % (v/v) *Pharmalyte* (pH 3 - 10), (*Pharmacia*, Uppsala, Sweden). In order to remove any charged compounds and before addition of *Pharmalytes*, gel solution was incubated for 10 min with an appropriate amount of a mixed bed ion-exchanger *Serdolit MB* (*Serva*, Heidelberg, Germany). IEF was conducted without prefocusing for 4 h. Voltage was 200 V during the first hour, and 600 V for the rest three hours. Prior to electrophoresis extruded tube gels were equilibrated for 15 min in running buffer containing 2 % (m/v) SDS, 30 % (v/v) glycerol, 8 M urea and tracking dye. The tube gel was then applied to the second dimension gel over a tin layer of 1 % (m/v) molten agarose. SDS-PAGE was performed in 10 % gel at 20 mA/gel constant current until the tracking dye reached bottom of the slab gel. Gels were fixed in 50 cm^3 of methanol:acetic acid:H_2O (40:10:50, v/v/v) solution for 30 min and silver stained overnight according Switzer *et al.* (1979) with some modifications. Fixed gels were washed 3 × 10 min with distilled water; sensitised for 30 min in a solution containing 30 % (v/v) ethanol, 6.8 % (m/v) sodium acetate, 0.2 % (m/v) sodium thiosulfate, 0.5 % (v/v) glutaraldehyde; washed 3 times in distilled water; stained for 30 min with 0.1 % (m/v) $AgNO_3$, 0.02 % (v/v) formaldehyde; and developed with 2.5 % (m/v) Na_2CO_3, 0.0004 % (m/v) $Na_2S_2O_3$, 0.01 % (v/v) formaldehyde. The reaction was stopped by transferring gels to 1 % (m/v) glycine solution. Stained gels were dried, photographed and scanned on flatbed scanner at 150 dpi (*Astra 1220P, UMAX*, Taiwan, China).

No significant differences between polypeptide profiles of control and SA-treated leaves were observed after 1-D PAGE (data not presented). Obviously, short-term treatment with SA did not trigger abundant expression or inhibition of soluble proteins like other physiologically active compounds, such as methyl-jasmonate (Metodiev 1998).

In order to analyze any minor changes in leaf polypeptide composition occurring upon 24-h treatment of barley plants with SA, we used two-dimensional SDS-PAGE. As a first step, we optimized the standard 2-D PAGE protocol (O'Farrell 1975) for separation of crude leaf extracts. The following modifications were found applicable for this purpose: *1)* using a non-denaturing detergent *CHAPS* instead of *Nonidet P40* for both gel and sample preparation, *2)* performing IEF without prefocusing, initially at 200 V (1 h) and then at 600 V (3 h), and *3)* separation of proteins in the second dimension without stacking gel. The range of protein focusing depended on the type of *Pharmalyte* used. When using *Pharmalyte 3-10* alone, we achieved an effective range of pH 3.5 - 9.0 for protein focusing. This was useful for separation of alkaline proteins (polypeptide profiles not presented). When mixing *Pharmalyte 5-8* and *Pharmalyte 3-10* (4:1, v:v) a non-linear pH gradient 4.0 - 8.0 was obtained (Fig. 1). In both cases protein separation did not suffer from a gradient drift which is commonly associated with longer times for isoelectric focusing applied for gels with standard dimensions

After 2-D PAGE at least 300 individual spots could be detected when 20 - 30 μg of proteins were loaded and gels were silver stained (Fig. 1). Although silver staining of proteins is not quantitative, the pair-wise comparison of the spots allowed for a quasi-quantitative assessment of SA impact on the abundance of particular spots. As pointed out by Switzer *et al.* (1979), proteins have different specificity to the silver stain but when protein amount is within the range 1 - 100 ng each individual

polypeptide appears as a spot stained proportionally to its quantity. Also, spot area represents an additional measure for polypeptide abundance.

On a map of 2-D electrophoretic pattern of soluble proteins from barley leaves (Fig. 2) individual polypeptides (PP) are shown as ellipses and described by their molecular mass in kD (first number) and pI (second number). Each ellipse area is proportional to the spot "volume", *i.e.* integrated intensity of the staining. The expression of a particular polypeptide was considered affected upon SA treatment when integrated intensity of the staining changed at least 20 %.

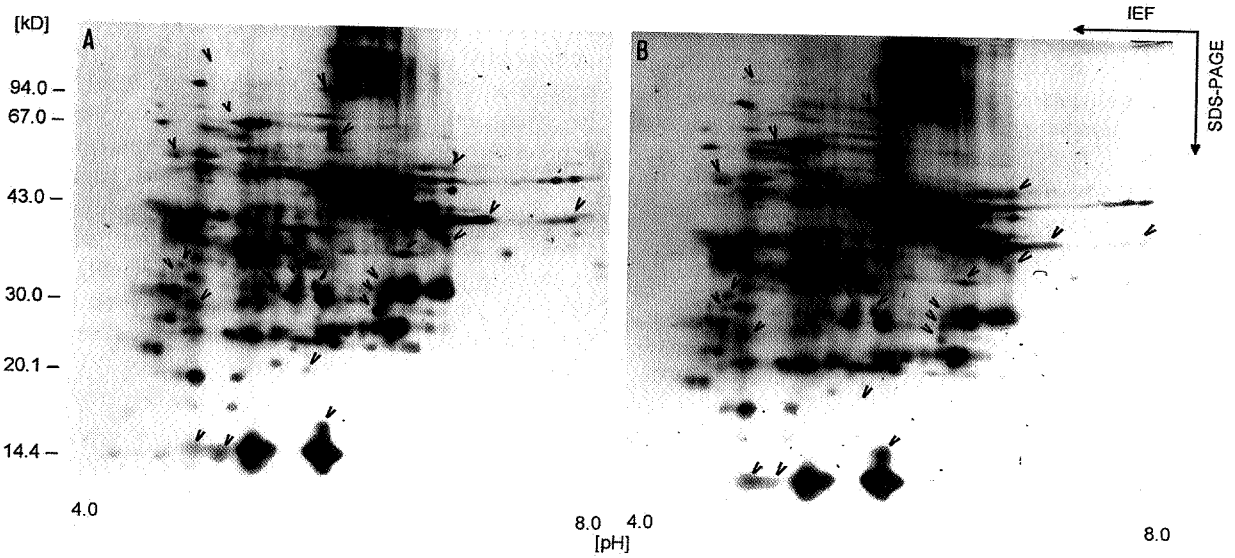

Fig. 1. Two-dimensional profile of soluble proteins extracted from barley leaves. *A* - control leaves, *B* - leaves treated 24 h with SA. IEF within pH range 4.0 - 8.0. SDS-PAGE was performed in 10 % gel and polypeptides visualized by silver staining. Molecular mass standards are present on the left. *Arrows* indicate changes in polypeptide amounts that change upon SA treatment.

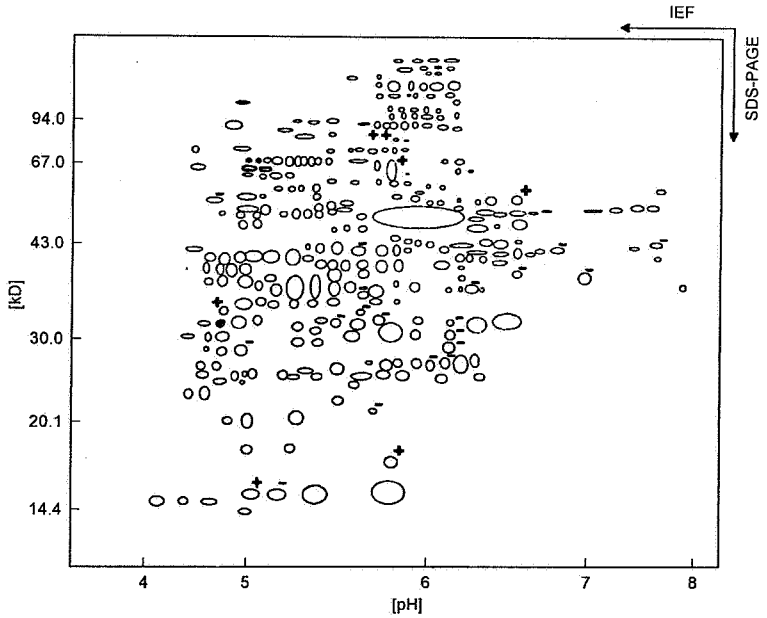

Fig. 2. Composite map of two-dimensional profile of soluble proteins extracted from barley leaves. The map is representative from 10 gels scanned at 150 dpi. Polypeptides which amounts change upon SA treatment are indicated as follows: increased - by "plus", decreased - by "minus", induced - in black.

We detected 19 polypeptides which amount decreased upon SA treatment. These were (indicated by "minus" in Fig. 2): PP 15/5.1; PP 21/5.8; PP 27.2/6.0; PP 27.2/6.1; PP 28.5/5.0; PP 29.2/6.1; PP 31/6.1; PP 32/6.1; PP 32/5.5; PP 34/5.9; PP 35/5.6; PP 37/5.6; PP 37/6.3; PP 38/7.0; PP 41/6.6; PP 47/6.8; PP 48/5.7; PP 48/7.9; PP 57.3/4.7. It should be noted that the negative effect of SA on these particular polypeptides was very strong.

Most of them (PP 15/5.1; PP 27/6.1; PP 28.5/5.0; PP 29.2/6.1; PP 31/6.1; PP 32/5.5; PP 34/5.9; PP 35/5.6; PP 37/6.3; PP 38/7.0; PP 41/6.6; PP 47/6.8; PP 57.3/4.7) had twice lower amounts while others (PP 21/5.8, PP 32/6.1 and PP 48/7.9) were almost undetectable in treated leaves (Fig. 1). The results suggested that expression of several soluble proteins was down-regulated within 24 h of SA application. Other proteins appeared to be up-regulated upon SA treatment since there were polypeptides which amount considerably increased in comparison to the control (indicated by "plus" in Fig. 2). These were PP 15/5.0; PP 17/5.9; PP 35/4.8; PP 54/6.6; PP 60.8/5.9; PP 87/5.8; PP 87/5.85 (Fig. 1). We distinguished 4 polypeptides as potentially newly induced in SA-treated barley leaves (indicated in black in Fig. 2), namely PP 32/4.8; PP 70/5.0; PP 70/5.05; and PP 96/5.0. Polypeptides PP 70/5.0 and PP 70/5.05 possessed same mobility on SDS-PAGE and slightly differed with respect to pI. This could reflect post-translational modification of a protein encoded by SA-inducible gene. Since the shift was toward acidic pH, we could speculate that 70 kD barley protein undergoes phosphorylation. Protein phosphorylation is involved in diverse plant stress responses and signalling pathways.

In this study we applied an optimized protocol for 2-D SDS-PAGE to analyze salicylic acid-induced changes in the soluble protein composition of barley leaves. The optimization allowed for sensitive and reproducible separation of crude leaf protein extracts. It was effective in characterization of minor alterations in protein expression upon SA treatment. The results showed that SA treatment led simultaneously to down regulation of the expression of several proteins and up-regulation of others. Although the lack of proteome information concerning plants does not allow us to speculate about the nature of the affected polypeptides, there is no doubt that SA mediated effects on protein expression deserve attention and further evaluation.

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