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LINK BETWEEN CYTOKININS, OXIDATIVE STRESS AND JASMONIC ACID AS REVEALED BY PROTEOME ANALYSIS IN *ARABIDOPSIS*

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I. Introduction

Cytokinins (CKs) regulate diverse developmental processes in plants. The molecular mechanisms of CK action are currently the subject of intense research. We analyzed changes in the *Arabidopsis* proteome caused by increase in endogenous CKs which was achieved by the employment of an improved pOp-*ipt*/LhGR transgenic system in which the production of CKs is inducible by dexamethasone (DEX) (Craft et al., 2005).

II. Results

1. Plant material



Fig. 1: Wild-type Columbia 0 (Col; control) and a transgenic pOp-*ipt*/LhGR line (*ipt*) grown for 15 days on MS medium in the absence and presence of 2.5 mM DEX, at 16h light/8h dark; 21 °C day/19 °C night; light intensity 117 mmol.m⁻².s⁻¹.

2. Quantitative analysis of CKs

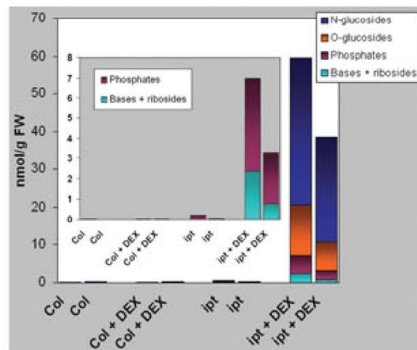


Fig. 2: CKs extracted by methanol/chloroform/acetic acid and purified on RP-C18 columns were quantified by LC/MS using deuterated CKs as internal standards. Samples were analysed in duplicates.

3. Two-dimensional (2-D) electrophoresis

2-D electrophoresis was performed in a classical set-up (IPG strips pH 4-7; 500 mg protein/strip; 11% SDS-PA gel;

colloidal CBB-G250 staining). Analysis of gel images in triplicates and subsequent statistical evaluation revealed significant differences between protein maps from DEX treated and non-treated pOp-*ipt*/LhGR plants (Fig. 3). No significant effects of DEX on wild-type protein maps were observed

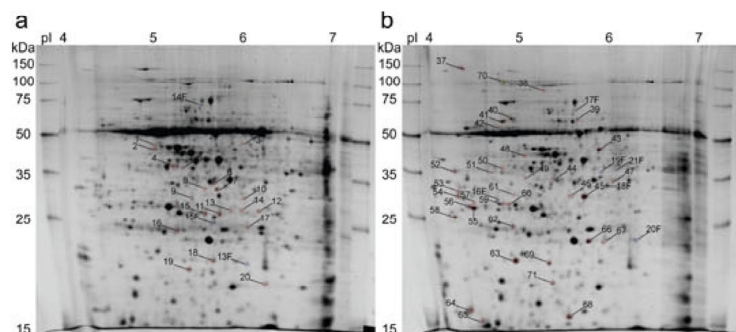


Fig. 3: Protein maps of pOp-*ipt*/LhGR plants grown in the absence (a) and presence (b) of 2.5 mM DEX; numbers indicate the significantly overexpressed spots.

4. Protein identification and functional classification

Total of 62 differentially expressed spots were subjected to MALDI-TOF MS analysis; 58 of them were characterized: 2 specific, 35 upregulated, 19 downregulated and 2 absent in gels from DEX treated pOp-*ipt*/LhGR plants (Table 1); and classified into functional categories (Fig. 4).

Table 1: Abbreviated list of differentially expressed proteins

| Regulation ^a | p ^b | Spot ^c | Protein ^d | Accession No. ^e | MOWSE score ^f | Seq. cover ^g |
|-------------------------|----------------|-------------------|--|----------------------------|--------------------------|-------------------------|
| Up | 0.001 | 45 | Jasmonate inducible protein isolog | O04314 | 266 | 67% |
| | 0.01 | 18F | 2'-hydroxyisoflavone reductase | P52577 | 112 | 37% |
| | 0.01 | 37 | Cor78 protein | Q39060 | 273 | 35% |
| | 0.05 | 20F | Glutathione-S-transferase | P42760 | 115 | 46% |
| | 0.05 | 43 | S-adenosylmethionine synthase | Q8LUT2 | 338 | 67% |
| | 0.05 | 61 | Phosphoribosylformylglycinamide cyclo-ligase precursor | Q05728 | 115 | 20% |
| | 0.05 | 66 | GSH-dependent dehydroascorbate reductase | Q9FWR4 | 289 | 83% |
| Down | 0.01 | 13 | L-ascorbate peroxidase | Q9STM9 | 231 | 50% |
| | 0.05 | 10 | Probable ethylene-inducible protein | O80448 | 207 | 46% |

Each entry contains: ^aType of regulation by DEX induction; ^bSignificance of quantitative change; ^cSpot number referring to that in Fig. 3; ^dBest matching result of Mascot search; ^eSwiss-Prot accession number; ^fSignificance of protein identification (p < 0.05, cut-off level = 85); ^gPercentage of protein sequence covered by MALDI-TOF MS analysis.

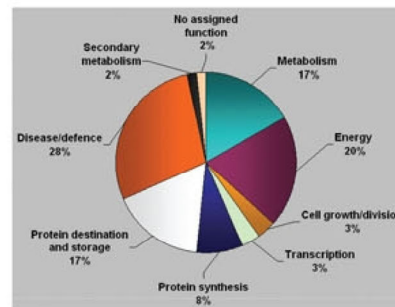


Fig. 4: Assignment of the differentially expressed proteins to functional categories according to Bevan et al. (1998).

Validity of results from 2-D analysis such as the high proportion of defence/oxidative stress related proteins in induced plants was further examined by the following experiments:

5. Quantitative analysis of jasmonic acid (JA)

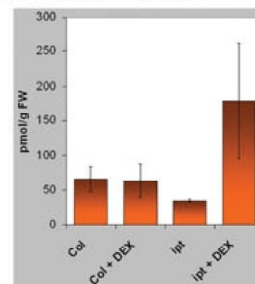


Fig. 5: Content of JA as quantified by LC/MS; samples were analyzed in triplicates.

6. Localization of hydrogen peroxide

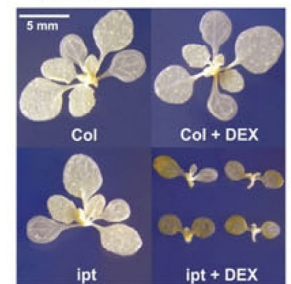


Fig. 6: Chromogenic detection of H₂O₂ in plant tissues by diaminobenzidine (DAB) staining (2.5 mM/2 h).

7. Assays for antioxidant enzymes

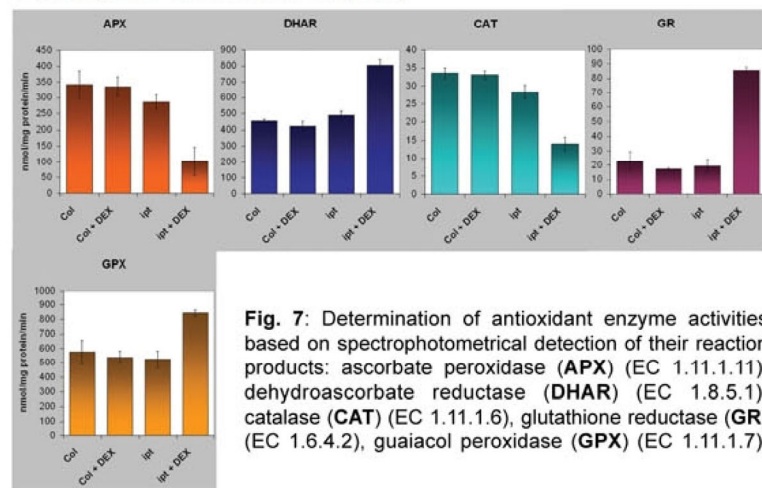


Fig. 7: Determination of antioxidant enzyme activities based on spectrophotometrical detection of their reaction products: ascorbate peroxidase (APX) (EC 1.11.1.11), dehydroascorbate reductase (DHAR) (EC 1.8.5.1), catalase (CAT) (EC 1.11.1.6), glutathione reductase (GR) (EC 1.6.4.2), guaiacol peroxidase (GPX) (EC 1.11.1.7).

III. Conclusions

- Image analysis of 2-D gels from seedlings with elevated CK levels and controls revealed significant differences in protein expression.
- High proportion of the differentially expressed proteins were related to oxidative stress and defense mechanisms including JA inducible proteins.
- Subsequent analyses revealed increased levels of hydrogen peroxide and JA in seedlings with elevated CKs together with significant changes in activity of several antioxidant enzymes.
- The data demonstrate, for the first time, a link between CKs and oxidative stress at the proteome level, implying cross-talk between CKs and JA in planta.

References:

Bevan M, et al. (1998) *Nature* 391:485-488
 Craft J, et al. (2005) *Plant J* 41:899-918

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