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Cytokinin in action is linked to oxidative stress as shown 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

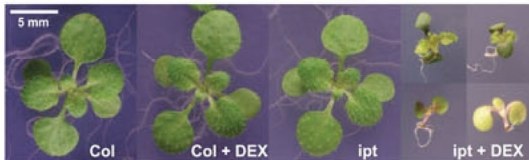


Fig. 1: Wild-type Columbia 0 (Col; control) and a transgenic pOp-*ipt*/LhGR line (*ipt*) grown for 15 days on MS medium in the absence and presence of 2.5 μ M DEX, at 16h light/8h dark; 21 °C day/19 °C night; light intensity 117 μ mol.m⁻².s⁻¹.

2. Quantitative analysis of CKs

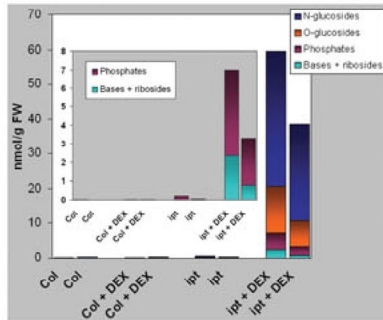


Fig. 2: CKs extracted by methanol/chloroform/acetic acid and purified on RP-C18 columns were quantified by LC/MS using deuterated CKs as internal standards. Samples were analysed in duplicates.

3. Two-dimensional (2-D) electrophoresis

2-D electrophoresis was performed as follows: IPG strips pH 4-7; 500 μ g protein/strip; 11% SDS-PA gel; colloidal Coomassie 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 seedlings (Fig. 3). No significant effects of DEX on wild-type protein maps were observed.

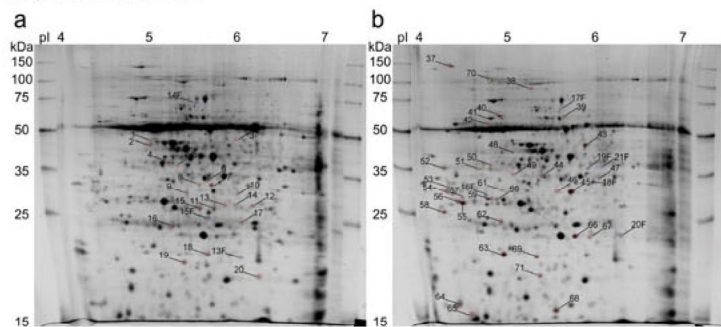


Fig. 3: Protein maps of pOp-*ipt*/LhGR seedlings grown in the absence (a) and presence (b) of 2.5 μ M DEX; numbers indicate the significantly overexpressed spots.

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). The identified proteins were classified into functional categories (Fig. 4).

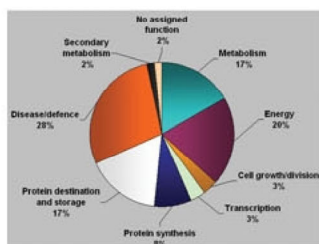


Fig. 4: Assignment of the differentially expressed proteins to functional categories according to Bevan et al. (1998).

Regulation	Spot	Protein
Up	45	Jasmonate inducible protein isolog
	18F	2'-hydroxyisoflavone reductase
	37	Cor78 protein
	20F	Glutathione-S-transferase
	43	S-adenosylmethionine synthase
	66	GSH-dependent dehydroascorbate reductase
Down	13	L-ascorbate peroxidase
	10	Probable ethylene-inducible protein

Table 1: Abbreviated list of differentially expressed proteins.

Validity of 2-D analysis results, particularly the high proportion of defence and oxidative stress related proteins, was examined by the following experiments:

5. Quantitative analysis of jasmonic acid (JA)

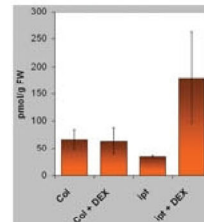


Fig. 5: Content of JA as quantified by LC/MS.

6. Localization of hydrogen peroxide

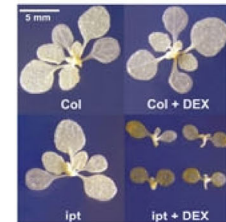


Fig. 6: Chromogenic detection of H₂O₂ in plant tissues by diaminobenzidine (DAB) staining (2.5 mM/2 h).

7. Assays for antioxidant enzymes

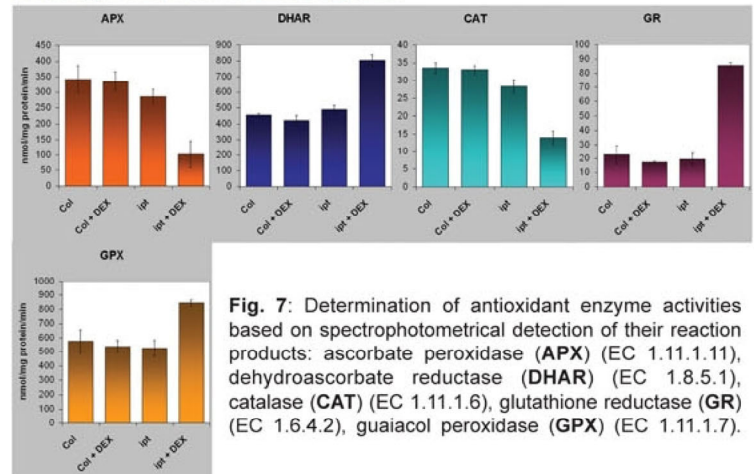


Fig. 7: Determination of antioxidant enzyme activities based on spectrophotometrical detection of their reaction products: ascorbate peroxidase (APX) (EC 1.11.1.11), dehydroascorbate reductase (DHAR) (EC 1.8.5.1), catalase (CAT) (EC 1.11.1.6), glutathione reductase (GR) (EC 1.6.4.2), guaiacol peroxidase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

8. Reduced glutathion (GSH) treatment

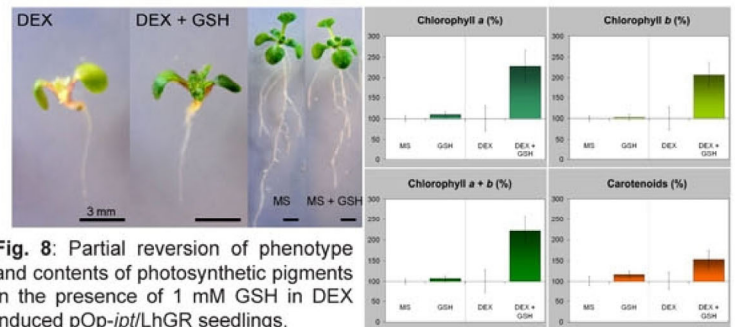


Fig. 8: Partial reversion of phenotype and contents of photosynthetic pigments in the presence of 1 mM GSH in DEX induced pOp-*ipt*/LhGR seedlings.

III. Summary

- 2-D electrophoresis as a top-down approach is a suitable tool for identifying candidates related to CK action on the protein level.
- 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.
- Application of reduced glutathion, an antioxidant, partially reversed the adverse effects caused by high CK levels (i.e. retarded development and contents of photosynthetic pigments).
- The data demonstrate 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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