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The effects of putrescine are partly overlapping with osmotic stress processes in wheat

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ABSTRACT

Polyamine metabolism is in relation with several metabolic pathways and linked with plant hormones or signalling molecules; in addition polyamines may modulate the up- or down-regulation of gene expression. However the precise mechanism by which polyamines act at the transcription level is still unclear. In the present study the modifying effect of putrescine pre-treatment has been investigated using the microarray transcriptome profile analysis under the conditions where exogenous putrescine alleviated osmotic stress in wheat plants. Pretreatment with putrescine induced the unique expression of various general stress-related genes. Although there were obvious differences between the effects of putrescine and polyethylene glycol treatments, there was also a remarkable overlap between the effects of putrescine and osmotic stress responses in wheat plants, suggesting that putrescine has already induced acclimation processes under control conditions. The fatty acid composition in certain lipid fractions and the antioxidant enzyme activities have also been specifically changed under osmotic stress conditions or after treatment with putrescine.

1. Introduction

Polyamines (PAs) are aliphatic amines, which can be found in relatively high amounts in all living cells. The most abundant PAs in plants are putrescine (PUT), spermidine and spermine. Involvement of PAs in plant responses to environmental stimuli has been demonstrated by several studies. The protective effects of elevated endogenous level (either due to exogenous PA treatment or to transgenic modification) have been reported in several plant species under various stress conditions [1–11]. Besides their direct protective role PAs regulate fundamental cellular processes as signalling molecules, suggesting that abiotic stress tolerance is mainly influenced via signalling processes rather than by their accumulation [12]. Although some studies have been published on this topic [5–6,13–18], the precise mechanism by which PAs act at the transcription level is still unclear. In a recent review it was suggested that a group of genes that increase their translation in the presence of PAs, so called “PA modulon” [19] in certain cases caused by an increase in their transcription factors. In addition, although PAs are usually considered as a family of similar molecules, different PAs may have different effects and the protective effect may vary as a function of the type of treatment.

The beneficial effect of putrescine (PUT) pre-treatment, added to the hydroponic solution, against PEG-induced osmotic stress has been demonstrated earlier in wheat and maize plants [11]. The results demonstrated that fine tuning in the PA pool is important for PA signalling, which influences the hormonal balance required. As more pronounced positive effects of PUT treatment were observed in wheat than in maize [11], in the present work only the wheat genotype was used as a continuation of the previous study in order to reveal a more detailed explanation for the background mechanism and processes behind the protective effect. The identification of polyamine-regulated downstream targets, such as polyamine-responsive elements and the corresponding transacting protein factors has opened up new possibilities to investigate the function of individual polyamines at transcriptional and translational levels. To our knowledge, this is the first report on the comparison of leaf gene expression profiling in response to PUT application under normal conditions and in the case of osmotic stress in wheat. Furthermore, changes in lipid composition and antioxidant

Abbreviations: APX, ascorbate peroxidase; DEG, Differentially expressed genes; DGDG, digalactosyldiacylglycerol; POD, guaiacol peroxidase; GR, glutathione reductase; MGDG, monogalactosyldiacylglycerol; PA, polyamine; PE, phosphatidylethanolamine; PEG, polyethylene glycol; PG, phosphatidylglycerol; PUT, putrescine; t16:1, trans-Δ^3-hexadecanoic acid; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid

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capacity have also been investigated. To our knowledge this is the first report on the comparison of the leaf gene expression profiling in response to putrescine under normal conditions and in the case of osmotic stress in wheat.

2. Materials and methods

2.1. Plant material and growth conditions

Wheat (*Triticum aestivum* L. TC33) (Thatcher-based near-isogenic line, TC33: Thatcher*6/P.I.58548) plants were used in the experiments. After 3 days of germination between moistened filter papers at 20 °C in the dark, 12 seedlings/plastic container were grown in modified Hoagland solution [20] at 20/18 °C with 16/8-h light/dark periodicity and 75% relative humidity in a Conviron GB-48 plant growth chamber (Controlled Environments Ltd, Winnipeg, Canada). The photosynthetic photon flux density (PPFD) was 250 $\mu mol\ m^{-2}\ s^{-1}$. The plant growth solution was changed every two days.

The experimental design was performed as in the previous experiment [11]. Briefly, after 7 days of growth in Hoagland solution, the seedlings were either grown further under control growth conditions in hydroponic solution or treated with 0.5 mM PUT hydroponically for 7 days. After this, the roots were washed in distilled water, then half of each group was grown further under control conditions (control); in the case of PUT-pretreated plants this functioned as a recovery period (PUT pre-treated). The other half of each group was treated with 15% PEG-6000 for 5 days (PEG or PUT-pretreated + PEG). Samples for microarray analysis were collected 24 h after the plants were treated with PEG or moved to control conditions either pre-treated with PUT or not. Samples for other analyses were collected at the end of the PEG treatment for both stressed and non-stressed plants.

2.2. Microarray analysis

For the microarray experiment three biological replicates were harvested and three technical replicates were isolated from each sample (each consisted of seven plants). RNA was isolated using an RNEasy Plant Mini Kit (Qiagen) and the samples were treated with DNase I (Qiagen) according to the manufacturer's instructions. The RNA Integrity Number (RIN) of the samples was determined with an Agilent BioAnalyzer. After assessing the RNA quality, equal amount of RNA samples with RIN > 8 were pooled and used for cRNA amplification. The RNA amplification and labelling procedure were accomplished according to the manufacturer's recommendations (Agilent). The cRNA of three biological replicates labelled with biotin were hybridized to the Agilent 4×44 K Wheat Chip. The fold change (FC) values of the samples were compared for C vs PUT, C vs PEG, C vs PUT + PEG, PEG vs PUT, PUT vs PUT + PEG and PEG vs PUT + PEG in a simple loop design. Genes with logFC > 2 and $P > 0.05$ were considered as potentially differentially expressed genes (DEG).

2.3. Quantitative real-time PCR (qRT-PCR) analysis

The microarray analysis was validated by qRT-PCR, using the same total RNA samples for microarray analysis and cDNA synthesis. cDNA was synthesized from 500 ng RNA with a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA). Eleven genes that showed significant changes in the microarray analysis were chosen for validation (HX136014, HX078083, CK162749, AJ414701, BU672278, HX086963, AK332789, CK211857, BJ220767, GH723108, U73214). The gene expression changes were examined using an ABI StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). The two internal control genes used for normalizing the variations in cDNA amounts were Ta542227 and Ta30797. The geometric mean of the internal control data was applied for normalization. The relative changes in gene expression were compared to the control group and quantified according to the comparative CT method (2-ΔΔCT method) [21]. (Suppl. Figs. 1–2)

2.4. Functional annotation of the microarray probes

The EST accessions of the DEG sequences of C vs PEG, C vs PUT, C vs PUT + PEG, PEG vs PUT, PUT vs PUT + PEG, PEG vs PUT + PEG were queried in Triticeae taxon using BLASTN. Afterwards, EST hits were investigated for protein function Triticeae and Arabideae taxa using BLASTN. Hits of the latter taxon ($e < 1e^{-4}$) were used for further analysis due to the higher number of known gene functions.

2.5. Venn-diagram comparison

Lists of DEGs of C vs PEG, C vs PUT, C vs PUT + PEG, PEG vs PUT, PUT vs PUT + PEG, PEG vs PUT + PEG with an $e < 1e^{-4}$ cut-off were compared in order to reveal the uniqueness and overlap of the individual genes using the InteractiVenn toolkit [22]. Up- and down-regulated cases were separately analysed.

2.6. Principal component analysis (PCA)

The similarity of gene expression data sets of each comparisons was analysed based on the significant logFC values ($p < 0.05$) of array probes using a *var-covar* matrix to PCA [23].

2.7. GO analysis

In order to ranking of gene functions in the three main ontologies (biological process, molecular function, cellular component), gene ontology (GO) analysis was carried out on the C vs PEG, C vs PUT, C vs PUT + PEG, PEG vs PUT, PUT vs PUT + PEG, PEG vs PUT + PEG comparisons. Singular enrichment analysis (SEA) was performed on the *Arabidopsis* protein accessions using the web-based agriGO toolkit [24] with the *Arabidopsis* gene model (TAIR10) as a reference, Fisher statistical test and the Yekutieli multiple comparison correction method (FDR under dependency) [25] at P-value < 0.05 level.

2.8. Enzyme assays

The isolation of antioxidant enzymes and measurements of their activities were performed as described in [20]. The ascorbate peroxidase (APX; EC 1.11.1.11.) activity was measured by monitoring the decrease in absorbance at 290 nm. The guaiacol peroxidase (POD; EC 1.11.1.7.) activity was determined at 470 nm and the glutathione reductase (GR; EC 1.6.4.2.) activity at 412 nm.

2.9. Separation of POD isoenzymes

POD isoenzymes were separated by native polyacrylamide gel electrophoresis [26] in a vertical Hoefer SE600 Ruby apparatus (GE Healthcare Life Sciences at) at 4 °C (200 V, 60 mA, 2.5 h). Total acrylamide concentration of 3.13% and 10% was applied for stacking and separating gels respectively. Bisacrylamide contents were 20% and 5% of total acrylamide concentration [27].

For the detection of isoenzymes the gels were soaked 2 times for 10 min in 0.2 M sodium acetate buffer (pH 5.5). After 20 min incubation in substrate solution (11 mM guaiacol, 0.63 mM $MnCl_2$ dissolved in 0.2 M acetate buffer (pH 5.5) the enzymatic reaction supervened by the addition of 5 mM H_2O_2. Since the total soluble protein content is not axiomaticallyevidently in correlation with translation of specific proteins − the amount of POD enzyme protein − thus the same volume of samples diluted in the same way was loaded on the gel. To detect activity changes of isoenzymes which are present in large and small quantities a unit and a fourfold unit of sample volume of each treatment

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