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. Pre-treatment 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 defence 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.

Keywords: gene expression; osmotic stress; putrescine; wheat;

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.

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 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 μmol 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 24h 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 4X44K 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. Fig 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 downregulated 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 minutes in 0.2 M sodium acetate buffer (pH 5.5). After 20 minutes 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 axiomatically\evidently 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 was loaded. After scan, density plots of grayscale images were measured by ImageJ. The protein content of the loaded samples was determined according to Bradford [28]. POD isoenzymes detected in the leaves and roots were indicated with LPOD and RPOD, respectively.

2.10. Lipid extraction and fatty acid analysis

The lipids were extracted as described by Pál et al. [29] using 1 g leaf samples. The various lipid classes were separated by TLC on silica gel plates, after which the fatty acids were transmethylated. The GC-FID analysis of fatty acid methyl esters (FAME) was carried out using a Shimadzu Model GC-FID2010 system (Shimadzu Co., Kyoto, Japan) fitted with an SP-2380 capillary column (30m×0.25mm I.D., df=0.20μm) (Supelco/Sigma-Aldrich Co., St. Louis, MO, USA). In this analysis, the injector and initial oven temperatures were 200 and 175°C, respectively. After 8 min, the oven temperature was increased to 240°C at a rate of 50°C min^{-1} and kept at this level for 5 min. Samples were injected in split mode with a 1/5 split ratio. The He carrier flow-rate was 37.8 cm sec^{-1}. Heptadecanoic acid was used as internal standard for the quantification.

The double bond index (DBI) was calculated from the mol% values using the following formula: DBI = 1*(%18:1)+2*(%18:2)+3*(%18:3). The percentage of unsaturation was calculated using the following formula: % unsat = (18:1+18:2+18:3)/ (16:0+16:1+18:0+18:1+18:2+18:3).

2.11. Statistical analysis

The experiments were repeated 3 times and representative data are shown. The results were the means of 5 measurements. The data were statistically evaluated using the standard deviation and *t-test* methods.

3. Results

3.1. Gene expression analyses

In order to better understand the effects of PUT treatment under control and osmotic stress conditions, a microarray analysis was carried out in plants treated with or without PUT, and with or without PEG. RNA was isolated from the leaves. In total, 44000 gene probes were examined on the chip. Genes as being significantly differentially expressed (DEG) when the logFC value was > 2 and P-value was < 0.05 were classified, which resulted 125 individual probe sequences in C vs. PEG, 47 in C vs. PUT, 122 in C vs. PUT+PEG, 99 in PEG vs. PUT, 110 in PUT vs. PUT+PEG and 71 in PEG vs. PUT+PEG.

The multivariate analysis was performed to show the similarity between the global gene expression patterns of each comparison (Fig. 1A-B). The biplot representation showed that variables C vs. PEG, PUT vs. PUT+PEG were similar for PC1 axis. Along axis of PC2, variables C vs. PUT, PEG vs. PUT+PEG and C vs. PUT+PEG showed similar heavy loadings. The data shows that PEG is the most significant variable, which causes distinction in gene expression and application of PEG caused a remarkable stress in plants. PUT was found to be a significant variable for PC2, and it has a lesser effect on the gene expression as compared to PEG. The PCA analysis shows that application of PEG by itself caused basic difference in the global gene expression as compared to all kind of treatment combinations of PUT.

After the functional annotation of the probes with an e-value < $1e^{-4}$ cut-off, the expression of 97 genes in C vs. PEG, 35 in C vs. PUT, 76 in C vs. PUT+PEG, 75 in PEG vs. PUT, 82 in PUT vs. PUT+PEG and 56 in PEG vs. PUT+PEG in wheat and 72 genes in C vs. PEG, 29 in C vs. PUT, 71 in C vs. PUT+PEG, 51 in PEG vs. PUT, 61 in PUT vs. PUT+PEG and 42 in PEG vs. PUT+PEG in Arabidopsis databases could be identified (Suppl. Table 1.). Table 1 shows selected PEG-, PUT- and PUT+PEG-induced gene functions focusing on unique

induction and/or relatively higher (close or >1) logFC values. No great difference could be observed in the number of up- and down-regulated genes expressed individually or commonly (Fig. 2.A-B). The list of gene functions of the Venn sets are separated to up- and down-regulated cases and found in Suppl. Table 2.

Altogether, 61+64 and 18+29 specifically induced genes with known function (down- + upregulated by PEG and PUT-treatments compared with the control plants, respectively). No genes were overlapping induced by both PEG and PUT. The effect of PUT in the PEG-treated plants was also significant: 27 and 44 genes were up-, or down-regulated, respectively. Not surprisingly, C vs. PUT+PEG shows a high level overlap with both C vs. PUT or C vs. PEG both in the case of up- and downregulated genes. However, 3 + 4 genes were found, which were differentially induced by C vs PUT but not in PEG vs PUT+PEG plants, indicating that the effects of PUT partially depends on the physiological state, in the present case the water status of the plants.

Gene function accessions of wheat could not be mapped to GO terms with satisfactory statistical support due to the low number of accessions investigated and the unknown functions in the query list. Most of the enrichment analysis, however, could be adequately performed when the Arabidopsis protein accessions were used. The results of GO analysis of the three GO classes (Fig. 3A biological process, 3B molecular function, and 3C cellular component) showed similar GO terms significantly overrepresented in most comparisons ($q<0.05$; q-value means the multiple-test adjusted p-value). According to the GO analyses, majorities of these genes are related to general cellular/metabolic processes or responses to environmental stimuli or stress-related processes concerning to biological processes. Significant terms are GO: 0009719 Response to endogenous stimulus, GO: 0042221 Response to chemical stimulus, GO: 0006950 Response to stress or GO: 0071702: Organic substance transport. Significant GO terms concerning molecular functions were the following: GO: 0003824 Catalytic activity, GO:

0016491 Oxidoreductase activity, GO: 0046914 Transition metal ion binding; and regarding them to cellular component are GO: 0012505 Endomembrane system.

3.2. Physiological and molecular changes

In order to characterise the overall effect of PUT and PEG treatments on wheat plants, the activation of the antioxidant enzymes together with the changes in the fatty acid composition of some lipid fractions were also determined.

3.2.1. Antioxidant enzymes

In the leaves GR and APX activities did not change after the applied treatments, while the activity of POD significantly increased after PEG alone (Table 2). In the roots GR and POD induced after PEG alone or after combined treatment (PUT pre-treated+PEG), while APX only after the combination of PUT and PEG treatments (Table 2).

As high induction of POD activity was found after the applied treatments, the isoenzyme analysis of PODs was performed by gel electrophoresis (Figure 4A-B). In leaves 5 protein bands were identified as POD isoenzymes (LPOD1-5). POD activities were very similar in control, PUT and PUT+PEG treated samples. Increased activity of all POD isoenzymes occurred only in PEG treated samples, whereas LPOD2 activity was highly increased. In roots 9 protein bands were separable by native PAGE, which showed the POD activity (RPOD1-9). The bands of RPOD6 and RPOD7 did not show demonstrative changes during the experiment. Compared to control all RPODs except for RPOD1, RPOD6 and RPOD7 gave weaker signal in PEG treated roots. Only RPOD2 and RPOD3 activity increased distinctly in PUT treated roots as compared to untreated roots. RPOD isoenzymes in the PUT+PEG treated samples were more active than in PEG treated samples. Moreover RPOD2, RPOD3 and RPOD5 activity in PUT+PEG treated samples was higher, than in control, however PUT stayed below.

3.2.2. Fatty acid composition

In order to analyse the fatty acid composition in different membrane fractions in the leaf, the following lipid fractions were examined: monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). The MGDG and DGDG fractions were characterized by a high proportion of linolenic acid (18:3), while in the PE and PG fractions palmitic acid (16:0) and 18:3 was the most abundant.

In the galactolipid fractions (MGDG and DGDG) the initial proportion of polyunsaturated 18:3 fatty acid was so high (95.44% in the MGDG and 86.64% in the DGDG) that under the present conditions the treatments applied did not influence it (Table 3). Nevertheless, the PEG treatment caused a significant decrease in the percentage of unsaturation and in the DBI of the MGDG fraction, especially due to the higher level of 18:0. The %unsat parameter in the DGDG was slightly but significantly lower in all the treated plants (PUT, PEG and PUT+PEG) than in the controls, resulted from the increase in mol% of 16:0 and 18:0 (Table 3).

In the phospholipid PE fraction osmotic stress and the combined treatment (PUT pre-treated + PEG) increased the proportion of 18:3 leading to an increase in DBI and unsaturation (Table 3). Changes in the fatty acid composition of the other phospholipid, PG showed partly different pattern after PEG or PUT+PEG treatments (Table 3). Although osmotic stress alone significantly increased the levels of 16:0, PUT pre-treatment or PUT pre-treated+PEG did not influenced it, but decreased 18:2. These differences resulted in slightly, but significantly higher DBI and unsaturation in case of PUT pre-treated+PEG treatment compared to PEG treatment alone. The amount of trans-Δ^3-hexadecanoic acid (t16:1), which was only detectable in the leaf PG, did not change significantly during the treatments (Table 3).

4. Discussion

Various drought stress responsive genes have been identified and characterized in crop plants (reviewed by [30]). Among these genes, for example those responsible for osmolyte biosynthesis, encoding water channels, ion transporters, heat shock proteins, and late embryogenesis abundant proteins are usually upregulated under drought conditions. Transcription factors, protein kinases and protein phosphatases, which are involved in signal transduction and influence expression of genes during stress responses were also induced. According to these the expression of genes both with metabolic and regulatory roles were altered.

Under the present experimental conditions PEG activated the enzymatic antioxidant system and caused alteration in the fatty acid composition of the membrane fractions. Furthermore PEG, among others, upregulated genes encoding an ethylene-forming enzyme, atypical CYS HIS rich thioredoxin 4, plasma membrane intrinsic protein 1C (aquaporin) and dehydrine indicating that PEG under these conditions caused remarkable stress. Abscisic acid-insensitive 5-like protein 6, a basic leucine zipper transcription factor, which responses to a variety of stresses [31], and putative AP2 domain transcriptional regulator, a member of dehydration-responsive element-binding proteins (DREBs), which is an important plant transcription factor and regulate the expression of many stress-inducible genes mostly in an ABA-independent manner and play a critical role in improving the abiotic stress tolerance of plants [32] were also induced. However, most of the induced genes are involved in general stress responses. For example, cold-regulated 413 plasma membrane protein 2 (COR413-PM2), which is a highly hydrophobic protein, and a member of the COR413 family that is regulated by water stress, light, and abscisic acid [33]. Pathogenesis-related protein 4 and pectinesterase inhibitor 12 have also been induced after PEG-induced osmotic stress, the latter one is an antifungal protein and may be involved in basal disease resistance, as well as in drought and

oxidative stress tolerance in plants [34]. The induction of aluminium-induced protein with YGL and LRDR motifs, which also responded to salt stress in *Populus* genotypes, and has a role in xylem developing and it is involved in cell wall metabolism [35] was also found in wheat plants treated with PEG.

PEG-treatment decreased the expression of the phytosulfokin receptor 1, which has a role in cell differentiation, organogenesis, proliferation, cellular longevity and growth. Overexpression of phytosulfokin receptor 1 (PSKR1) in Arabidopsis lines resulted in larger leaves than in wild type and caused delayed senescence [36]. Furthermore, phytosulfokin signalling suggested to attenuate stress responses via the suppression of salicylic acid (SA) signalling. It was also found that, pskr1 mutants accumulated higher levels of SA and SA-responsive pathogenesis related gene transcripts [37]. As it was previously reported under the same conditions osmotic stress increased the free SA content in the leaves . Also higher leaf SA content was detected, when PUT pre-treatment preceded PEG-induced osmotic stress compared to the control [11]. According to these our results also support the hypothesis that there is an antagonistic relationship between SA-dependent defence pathway and phytosulfokin signalling [37].

Similarly to our results, in recent studies drought induced up-regulation of ethylene biosynthesis in bentgrass [18] and in soybean [38]. Also in bentgrass the expression of a gene encoding dehydrin RAB15 was up-regulated in response to drought stress, which is an ABA and drought induced molecular chaperone [18]. Besides genes involved in the synthesis of plant hormones, signalling pathways, synthesis of osmoprotectants, those that encode the members of the antioxidant system has been also reported to alter upon water deficit [39]. The abundances of peroxidases (PODs) were also increased in the leaves of drought-tolerant rice cultivar [40]. Moreover, the expression of POD genes was also increased in *Tamarix hispida* under drought stress [41], while POD isoenzymes induced differently in Ramonda leaves under drought stress

[42]. Under the present conditions, osmotic stress specifically induced a POD isoenzyme in the leaves, called LPOD2.

Earlier microarray transcriptome profile analysis of transgenic Arabidopsis lines overexpressing the arginine decarboxylase 2 (ADC2), the gene encoding the enzyme responsible for the synthesis of PUT from arginine, with elevated PUT levels revealed that these lines are resistant for freezing and drought stress [3; 43-44], and they also upregulated genes involved in responses to biotic and abiotic stresses. Expression changes were also observed for several members of the S-adenosyl-L-methionine:carboxyl-methyltransferase protein family, involved in SA biosynthesis in ADC overexpressing transgenic Arabidopsis plant with increased PUT content [5]. Genetic manipulation of PUT biosynthesis (overexpression of the ornithine decarboxylase: ODC, the gene encoding the enzymes responsible for the other pathway of PUT synthesis from ornithine) resulted in high putrescine producing cell line of poplar. Functional clustering analysis revealed that in these lines genes associated with photosynthesis, glycolysis and methionine biosynthesis, genes encoding glutathione S-transferase and acetyl-transferase, peroxidases and pathogenesis-related protein, furthermore genes involved in phenolic metabolism were down regulated, while genes related to polysaccharide catabolism, genes encoding defence-related chitinases, heat-shock proteins, cold-stress protein and metallothioneins were up regulated [45]. Under the same as the present conditions 0.5 mM PUT pre-treatment increased the shoot fresh and dry weight and the net photosynthesis, indicating the general roborative effect of the treatment [11]. PUT also upregulated several similar genes as it was found in the case of PEG treatment induced for example dehydrin, ethylene-forming enzyme, aluminium-induced protein with YGL and LRDR motifs, RmlC-like cupins superfamily protein and germin-like protein subfamily T member 1. RmlC-like cupins superfamily comprises several families with members performing diverse functions ranging from enzymatic activities like dioxygenases, decarboxylases, hydrolases,

isomerases and epimerases to non-enzymatic functions such as binding to auxin, nuclear transcription factors and seed storage [46]. While, germin-like superfamily members are ubiquitously expressed in various plant species and play important roles in plant development and defence. Germine-like protein genes have been reported to have its unique response to various abiotic stresses (including salt, H_2O_2 stress and wound), biotic stresses and plant hormone stimulations (including salicylic acid and abscisic acid treatments) in peanuts [47]. Increased expression of S-adenosyl-L-methionine-dependent methyltransferases, which has role in recycling of cytosolic SAM [48] has also been detected. However unique induction of the genes related to arogenate dehydratase 3, electron transfer flavoprotein ubiquinone oxide reductase (ETFQO), metallothionein 3 and 6-phosphogluconate dehydrogenase decarboxylating 1 was also found after PUT pre-treatment compared to the control and PEG treatment alone. Arogenate dehydratase catalyses the last steps of the biosynthesis of phenylalanine in plants, and its involvement in coordinating the homeostasis of reactive oxygen species and cotyledon development in etiolated Arabidopsis has been recently demonstrated [49]. The essential role of ETFQO in Arabidopsis during long-term dark-induced carbohydrate deprivation has been also reported [50]. The synthesis of metallothioneins has been reported to induce not only after heavy metal stress, but during osmotic stress and after hormone treatment [51-52]. Plastidic forms of 6-phosphogluconate dehydrogenase (G6PDH) involved in the oxidative pentose phosphate pathway, which provides NADPH for reductive biosyntheses and for protection against oxidative stress, and pentoses for synthesis of nucleotides and sugar phosphates for the shikimate pathway [53]. The induction of the chloroplastic enzymes, such as 6-phosphogluconate dehydrogenase decarboxylating 1 and arogenate dehydratase 3 suggests that PUT pre-treatment activates the pentose phosphate and shikimate-chorismate pathways in the chloroplasts as part of the acclimation process. In addition the up-regulation of cytosolic NADP-dependent isocitrate dehydrogenase, which has been reported to contribute to

redox homeostasis and the regulation of pathogen responses in Arabidopsis leaves, has been also induced under the present condition after PUT pre-treatment [54]. Interestingly, when compared the list of genes induced by PEG, PUT or PUT pre-treated + PEG, a partly similar pattern was found for the combined treatment, as higher expression was found for cold-regulated 413 plasma membrane protein 2, dehydrine family protein, pathogenesis-related 4, aquaporin, putative non-LTR retroelement reverse transcriptase (abscisic stress-ripening protein), metallothionein 3, S-adenosyl-L-methionine-dependent methyltransferases superfamily protein and sulfurtransferase 18, as it was described for PEG or PUT treatment.

Although, upregulation of peroxidase superfamily protein was found in PUT pre-treated plants, at the end of the experiment similar activity of the antioxidant enzymes, and similar pattern of POD isoenzymes was found in PUT-pre-treated plant as in the controls. After PUT pre-treated+PEG treatment increased peroxidase expression was found; and the POD isoenzyme analysis showed that in the presence of PEG there is a great possibility that PUT pre-treatment has a role in keeping the POD activity around the control level.

PUT has also been reported to alter fatty acid composition in the thylakoid membrane, therefore may stabilise photosynthetic apparatus under salt stress [55]. In the present study a bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein (Dir1) [56] and PLAT/LH2 domain-containing lipoxygenase have been upregulated not only under osmotic stress, but after PUT pre-treatment, while the highest induction was found after PUT pre-treated + PEG treatment. The PLAT/LH2 domain-containing lipoxygenase also has a role in plant signalling, as it was reported to be induced by ABA, SA, jasmonate or wounding [57]. When fatty acid composition was investigated, it was found that although PUT treatment had a few specific effects, these differences were less substantial than the effects for osmotic stress (resulted in higher DBI in PE, but lower DBI in MGDG fractions) and they probably do not explain the previously observed protective effects of PUT.

In conclusion, the present study shows that pre-treatment of wheat plants with PUT induced the expression of various general stress related genes. Although there were obvious differences between the effects of PUT and PEG treatments, including expression of various genes, there was also a remarkable overlap between the effects of PEG and PUT.

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References

[1] N. Lakra, S.N. Mishra, D.B. Singh, P.C. Tomar, Exogenous putrescine effect on cation concentration in leaf of *Brassica juncea* seedlings subjected to Cd and Pb along with salinity stress. J. Environ. Biol. 27 (2006) 263-269. http://jeb.co.in/journal_issues/200604_apr06/paper_18.pdf

[2] J.C. Cuevas, R. López-Cobollo, R. Alcázar, X. Zarza, C. Koncz, T. Altabella, J. Salinas, A.F. Tiburcio, A. Ferrando, Putrescine is involved in *Arabidopsis* freezing tolerance and cold acclimation by regulating abscisic acid levels in response to low temperature, Plant Physiol. 148 (2008) 1094-1105. DOI: 10.1104/pp.108.122945

[3] R. Alcázar, T. Altabella, F. Marco, C. Bortolotti, M. Reymond, C. Koncz, C. Parrasco, A.F. Tiburcio, Polyamines: molecules with regulatory functions in plant abiotic stress tolerance, Planta 231 (2010) 1237-1249. DOI: 10.1007/s00425-010-1130-0

[4] C. Pandolfi, I. Pottosin, T. Cuin, S. Mancuso, S. Shabala, Specificity of polyamine effects on NaCl-induced ion flux kinetics and salt stress amelioration in plants, Plant and Cell Physiology 51 (2010) 422-434. DOI: 10.1093/pcp/pcq007

[5] F. Marco, R. Alcázar, A.F. Tiburcio, P. Carrasco, Interactions between polyamines and abiotic stress pathway responses unraveled by transcriptome analysis of polyamine overproducers, OMICS, 15 (2011) 775-781. DOI: 10.1089/omi.2011.0084

[6] B. Gupta, K. Gupta, D.N. Sengupta, Spermidine-mediated in vitro phosphorylation of transcriptional regulator OSBZ8 by SNF1-type serine/threonine protein kinase SAPK4 homolog in indica rice, Acta Physiol. Plant. 34 (2012) 1321-1336. DOI:10.1007/s11738-012-0929-7

[7] S. Mandal, A. Mandal, H.E. Johansson, A.V. Orjalo, M.H. Park, Depletion of cellular polyamines, spermidine and spermine, causes a total arrest in translation and growth in mammalian cells. Proc. Natl. Acad. Sci. 110 (2013) 2169-2174. DOI: 10.1073/pnas.1219002110

[8] I. Marcińska, I. Czyczyło-Mysza, E. Skrzypek, M. Grzesiak, F. Janowiak, M. Filek, M. Dziurka, K. Dziurka, P. Waligórski, K. Juzoń, K. Cyganek, S. Grzesiak, Alleviation of osmotic stress effects by exogenous application of salicylic or abscisic acid on wheat seedlings, Int. J. Mol. Sci. 14 (2013) 13171-13193. DOI: 10.3390/ijms140713171

[9] C. Kotakis, E. Theodoropoulou, K. Tassis, C. Oustamanolakis, N.E. Ioannidis, K. Kotzabasis, Putrescine, a fast-acting switch for tolerance against osmotic stress, J. Plant Physiol. 171 (2014) 48-51. DOI:10.1016/j.jplph.2013.09.015

[10] G. Szalai, M. Pál, T. Árendás, T. Janda, Priming seed with salicylic acid increases grain yield and modifies polyamine levels in maize. Cereal Res. Commun. 44 (2016) 537-548. DOI:10.1556/0806.44.2016.038

[11] G. Szalai, K. Janda, E. Darkó, T. Janda, V. Peeva, M. Pál, Comparative analysis of polyamine metabolism in wheat and maize plants, Plant Physiol. Biochem. (2017) DOI: 10.1016/j.plaphy.2017.01.012.

[12] M. Pál, G. Szalai, T. Janda, Speculation: Polyamines are important in abiotic stress signaling, Plant Sci. 237 (2015) 16-23. DOI:10.1016/j.plantsci.2015.05.003

[13] Y. Kasukabe, L. He, K. Nada, S. Misawa, I. Ihara, S. Tachibana, Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic Arabidopsis thaliana, Plant Cell Physiol. 45 (2004) 712-722. DOI:10.1093/pcp/pch083

[14] Z. Li, Y. Peng, X.Q. Zhang, X. Ma, L.K. Huang, Y.H. Yan, Exogenous spermidine improves seed germination of white clover under water stress via involvement in starch metabolism, antioxidant defenses and relevant gene expression, Molecules, 19 (2014a) 18003-18024. DOI: 10.3390/molecules191118003

[15] Z. Li, Y. Peng, X.Q. Zhang, M.H. Pan, X. Ma, L.K. Huang, Y.H. Yan, Exogenous spermidine improves water stress tolerance of white clover (*Trifolium repens* L.) involved in antioxidant defence, gene expression and proline metabolism, POJ, 7 (2014b) 517-526. DOI:10.3390/molecules191118003

[16] F.D. Espasandin, S.J. Maiale, P. Calzadilla, O.A. Ruiz, P.A. Sansberro, Transcriptional regulation of 9-cis-epoxycarotenoid dioxygenase (NCED) gene by putrescine accumulation positively modulates ABA synthesis and drought tolerance in *Lotus tenuis* plants, Plant Phys. Biochem. 76 (2014) 29-35. DOI:10.1016/j.plaphy.2013.12.018

[17] W. Tong, K. Yoshimoto, J. Kakehi, H. Motose, M. Niitsu, T. Takahashi, Thermospermine modulates expression of auxin-related genes in Arabidopsis, Front. Plant Sci. 5 (2014) 94. DOI: 10.3389/fpls.2014.00094

[18] Y. Ma, V. Shukla, E.B. Merewitz, Transcriptome analysis of creeping bentgrass exposed to drought stress and polyamine treatment. PLoS One. 26 (2017) e0175848. DOI: 10.1371/journal.pone.0175848

[19] L. Miller-Fleming, V. Olin-Sandoval, K. Campbell, M. Ralser, Remaining mysteries of molecular biology: The role of polyamines in the cell. J. Mol Biol 427 (2015) 3389-3406. DOI: 10.1016/j.jmb.2015.06.020

[20] M. Pál, E. Horváth, T. Janda, E. Páldi, G. Szalai, Cadmium stimulates the accumulation of salicylic acid and its putative precursors in maize (Zea mays L.) plants, Physiol. Plant. 125 (2005) 356-364. DOI: 10.1111/j.1399-3054.2005.00545.x

[21] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25(2001) 402-408. DOI: 10.1006/meth.2001.1262

[22] H. Heberle, G.V. Meirelles, F.R. da Silva, G.P. Telles, R. Minghim, InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams, BMC Bioinformatics 16 (2015) 169. DOI: 10.1186/s12859-015-0611-3

[23] Ř. Hammer, D.A.T. Harper, P.D. Ryan, PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4 (2001) 9pp. http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

[24] T. Tian, Y. Liu, H. Yan, Q. You, X. Yi, Z. Du, W. Xu, Z. Su, agriGO v2.0: a GO analysis toolkit for the agricultural community, 2017 update. Nucleic Acids Res. 45 (2017) gkx382. DOI: 10.1093/nar/gkx382

[25] Y. Benjamini, D. Yekutieli, The control of the false discovery rate in multiple testing under dependency, Ann Statist. 29 (2001) 1165-88. DOI:10.1214/aos/1013699998

[26] U. K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227(1970), 680-685. DOI: 10.1038/227680a0

[27] Z. Nagy, E. Németh, A. Guóth, L. Bona, B. Wodala, A. Pécsváradi, Metabolic indicators of drought stress tolerance in wheat: Glutamine synthetase isoenzymes and Rubisco. Plant Physiol. Biochem. 67 (2013) 48-54. DOI: 10.1016/j.plaphy.2013.03.001

[28] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254. DOI:10.1016/0003-2697(76)90527-3

[29] M. Pál, N. Leskó, T. Janda, E. Páldi, G. Szalai, Cadmium-induced changes in the membrane lipid composition of maize plants, Cereal Res. Commun. 35 (2007) 1631-1642. DOI:10.1556/CRC.35.2007.4.10

[30] R. Joshi, S.H. Wani, B. Singh, A. Bohra, Z.A. Dar, A.A. Lone, A. Pareek, S.L. Singla-Pareek, Transcription Factors and Plants Response to Drought Stress: Current Understanding and Future Directions, Frontiers in Plant Science. 7 (2016) 1029. DOI:10.3389/fpls.2016.01029.

[31] I.M. Brocard, T.J. Lynch, R.R. Finkelstein, Regulation and role of the Arabidopsis abscisic acid-insensitive 5 gene in abscisic acid, sugar, and stress response. Plant Physiol. 129 (2002) 1533-1543; DOI: 10.1104/pp.005793

[32] C. Lata, M. Prasad, Role of DREBs in regulation of abiotic stress responses in plants. J. Exp. Bot. 62 (2011) 4731–4748 DOI.org/10.1093/jxb/err210

[33] G. Breton, J. Danyluk, J.-B.F. Charron, F. Sarhan, Expression Profiling and Bioinformatic Analyses of a Novel Stress-Regulated Multispanning Transmembrane Protein Family from Cereals and Arabidopsis. Plant Physiol. 132 (2003) 64-74. DOI:10.1104/pp.102.015255.

[34] S.H. An, K.H. Sohn, H.W. Choi, I.S. Hwang, S.C. Lee, B.K..Hwang, Pepper pectin methylesterase inhibitor protein CaPMEI1 is required for antifungal activity, basal disease resistance and abiotic stress tolerance. Planta. 228 (2008) 61-78. DOI:10.1007/s00425-008-0719-z.

[35] D. Janz, S. Lautner, H. Wildhagen, K. Behnke, J.P. Schnitzler, H. Rennenberg, J. Fromm, A. Polle, Salt stress induces the formation of a novel type of ‘pressure wood’ in two

Populus species. New Phytologist, 194 (2012) 129-141. DOI: 10.1111/j.1469-8137.2011.03975.x

[36] Y. Matsubayashi, M. Ogawa, H. Kihara, M. Niwa, Y. Sakagami, Disruption and overexpression of Arabidopsis phytosulfokine receptor gene affects cellular longevity and potential for growth. Plant Physiol. 142 (2006) 45-53. DOI: 10.1104/pp.106.081109

[37] S. Mosher, B.Kemmerling, PSKR1 and PSY1R-mediated regulation of plant defense responses. Plant Signal. Behav. 8 (2013) e24119. DOI:10.4161/psb.24119.

[38] F.B.M. Arraes, M.A. Beneventi, M.E. Lisei-de-Sa, J.F.R. Paixao, E.V.S Albuquerque, S.R.R Marin, E. Purgatto, A.L. Nepomuceno, M.F. Grossi-de-Sa, Implications of ethylene biosynthesis and signaling in soybean drought stress tolerance, BMC Plant Biol. 15 (2015) 213-233. DOI: 10.1186/s12870-015-0597-z

[39] X. Wang, X. Cai, C. Xu, Q. Wang, S. Dai, Drought-Responsive Mechanisms in Plant Leaves Revealed by Proteomics. Komatsu S, Hossain Z, Int. J. Mol. Sci. 17 (2016) 1706. DOI: 10.3390/ijms17101706

[40] M.L. Raorane, I.M. Pabuayon, A.R. Varadarajan, S.K. Mutte, A. Kumar, A. Treumann, A. Kohli, Proteomic insights into the role of the large-effect QTL qDTY12.1 for rice yield under drought. Mol. Breed. 35 (2015) 1-14. DOI:10.1007/s11032-015-0321-6

[41] C.Q. Gao, Y.C. Wang, G.F. Liu, C. Wang, J. Jiang, C.P. Yang, Cloning of ten peroxidase (POD) genes from *Tamarix hispida* and characterization of their responses to abiotic stress, Plant Mol. Biol. Rep. 28 (2010) 77-89. DOI: 10.2174/138920291505141106102544

[42] S. Veljovic-Jovanovic, B. Kukavica, B. Stevanovic, F. Navari-Izzo, Senescence- and drought-related changes in peroxidase and superoxide dismutase isoforms in leaves of *Ramonda serbica*. J. Exp. Bot. 57 (2006) 1759-1768. DOI: 10.1093/jxb/erl007

[43] R. Alcázar, J.L. Garcia-Martinez, J.C. Cuevas, A.F. Tiburcio, T. Altabella, Overexpression of ADC2 in Arabidopsis induces dwarfism and late-flowering through GA deficiency, Plant J. 43 (2005) 425-436. DOI: 10.1111/j.1365-313X.2005.02465.x

[44] A.F. Tiburcio, T. Altabella, A. Ferrando, Plant with resistance to low temperature and method of production thereof. Spanish patent application (2009) WO2010/004070.

[45] A.F. Page, L.J. Cseke, R. Minocha, S.A. Turlapati, G.K. Podila, A. Ulanov, Z. Li, S.C. Minocha, Genetic manipulation of putrescine biosynthesis reprograms the cellular transcriptome and the metabolome. BMC Plant Biology. 16 (2016) 113. DOI:10.1186/s12870-016-0796-2.

[46] G. Agarwal, M. Rajavel, B. Gopal, N. Srinivasan, Structure-based phylogeny as a diagnostic for functional characterization of proteins with a cupin fold. PLoS One 4 (2009) e5736. DOI: 10.1371/journal.pone.0005736

[47] T. Wang, X. Chen, F. Zhu, H Li, L. Li, YQ. Yang, X. Chi, Characterization of Peanut Germin-Like Proteins, AhGLPs in Plant Development and Defense. Unver T, ed. PLoS ONE. 2013;8(4):e61722. DOI:10.1371/journal.pone.0061722.

[48] M. Sauter, B. Moffatt, M.C. Saechao, R. Hell, M. Wirtz, Methionine salvage and S-adenosylmethionine: essential links between sulfur, ethylene and polyamine biosynthesis, Biochem. J. 451 (2013) 145-154. DOI: 10.1042/BJ20121744

[49] A. Para, D.S. Muhammad, D.A. Orozco-Nunnelly, R. Memishi, S. Alvarez, M.J. Naldrett, K.M. Warpeha, The dehydratase ADT3 affects ROS homeostasis and cotyledon development, Plant Physiol. 172 (2016) 1045-1060; DOI: 10.1104/pp.16.00464

[50] K. Ishizaki, T.R. Larson, N.Schauer, A.R. Fernie, I.A. Graham, C.J. Leaver, The critical role of Arabidopsis electron-transfer flavoprotein:ubiquinone oxidoreductase during dark-induced starvation. Plant Cell, 17 (2005) 2587-2600. DOI: https://doi.org/10.1105/tpc.105.035162

[51] F. Moloudi, S. Navabpour, H. Soltanloo, S.S. Ramazanpour, H. Sadeghipour, Catalase and metallothionein genes expression analysis in wheat cultivars under drought stress condition, JPMB 1 (2013) 54-68. DOI: 10.22058/JPMB.2013.3262

[52] L. Yang, J. Wang, J. Yang, R. Schamber, N. Hu, S. Nair, L. Xiong, J. Ren, Antioxidant metallothionein alleviates endoplasmic reticulum stress-induced myocardial apoptosis and contractile dysfunction. Free Radic Res. 49 (2015) 1187-1198. DOI: 10.3109/10715762.2015.1013952

[53] K. Krepinsky, M. Plaumann, W. Martin, C. Schnarrenberger, Purification and cloning of chloroplast 6-phosphogluconate dehydrogenase from spinach. Cyanobacterial genes for chloroplast and cytosolic isoenzymes encoded in eukaryotic chromosomes. Eur J Biochem. 268 (2001) 2678-2686. DOI: 10.1046/j.1432-1327.2001.02154.x

[54] A. Mhamdi, C.Mauve, H. Gouia, P. Saindrenan, M. Hodges, Noctor G. Cytosolic NADP-dependent isocitrate dehydrogenase contributes to redox homeostasis and the regulation of pathogen responses in Arabidopsis leaves. Plant Cell Environ. 33 (2010) 1112-1123. DOI: 10.1111/j.1365-3040.2010.02133.x.

[55] S. Shu, S.R. Guo, L.Y. Yuan, A review: polyamines and photosynthesis, in Advances in Photosynthesis-Fundamental Aspects, M. M. Najafpour, Ed., InTech, Rijeka, Croatia, 2012. pp. 439-464. DOI: 10.5772/26875

[56] M.B. Lascombe, B. Bakan, N. Buhot, D. Marion, J.P. Blein, V. Larue, C. Lamb, T. Prangé, The structure of "defective in induced resistance" protein of Arabidopsis thaliana, DIR1, reveals a new type of lipid transfer protein. Protein Science : A Publication of the Protein Society. 17 (2008) 1522-1530. DOI: 10.1110/ps.035972.108

[57] S.T. Li, M. Zhang, C.H. Fu, S. Xie, Y. Zhang, L.J. Yu, Molecular Cloning and Characterization of Two 9-Lipoxygenase Genes from Taxus chinensis, Plant Molecular Biology Reporter, 30 (2012) 1283–1290. DOI:10.1007/s11105-012-0439-1

Table 1. Lists of gene functions induced by PEG or putrescine (PUT) or PEG+PUT (note: **bold letters** indicates unique functions between PEG- and PUT-induced lists and *italics* indicates unique functions between PEG- and PUT+PEG-induced lists).

PEG-induced	***PUT-induced***	***PUT+PEG-induced***
	6-phosphogluconate dehydrogenase, decarboxylating 1, chloroplastic [Arabidopsis lyrata subsp. lyrata] |XP_002886358|	
ABSCISIC ACID-INSENSITIVE 5-like protein 6 [Arabidopsis lyrata subsp. lyrata] |XP_020874468|		ABSCISIC ACID-INSENSITIVE 5-like protein 6 [Arabidopsis lyrata subsp. lyrata] |XP_020874468|
aluminum induced protein with YGL and LRDR motifs [Arabidopsis thaliana] >gi|7021730|gb|AAF35411.1|	aluminum induced protein with YGL and LRDR motifs [Arabidopsis thaliana] >gi|7021730|gb|AAF35411.1|	
	arogenate dehydratase 3, chloroplastic [Arabidopsis lyrata subsp. lyrata] |XP_002880966|	
atypical CYS HIS rich thioredoxin 4 [Arabidopsis thaliana] |NP_001117248|		
Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein [Arabidopsis thaliana] |NP_190966|	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein [Arabidopsis thaliana] |NP_190966|	
cold-regulated 413 plasma membrane protein 2 [Arabidopsis lyrata subsp. lyrata] |XP_002877776|		cold-regulated 413 plasma membrane protein 2 [Arabidopsis lyrata subsp. lyrata] |XP_002877776|
dehydrin family protein [Arabidopsis thaliana] |NP_190666|		dehydrin family protein [Arabidopsis thaliana] |NP_190666|
dehydrin Rab18 isoform X2 [Arabidopsis lyrata subsp. lyrata]|XP_020881942|	dehydrin Rab18 isoform X2 [Arabidopsis lyrata subsp. lyrata]|XP_020881942|	dehydrin Rab18 isoform X2 [Arabidopsis lyrata subsp. lyrata]|XP_020881942|
ethylene-forming enzyme [Arabidopsis thaliana] |NP_171994|	ethylene-forming enzyme [Arabidopsis thaliana] |NP_171994|	ethylene-forming enzyme [Arabidopsis thaliana] |NP_171994|
ethylene-forming enzyme [Arabidopsis thaliana]|CAA47251|	ethylene-forming enzyme [Arabidopsis thaliana]|CAA47251|	
	ETFQO [Arabidopsis thaliana]|OAP10842|	
germin-like protein subfamily T member 1 [Arabidopsis lyrata subsp. lyrata]|XP_020870018|	germin-like protein subfamily T member 1 [Arabidopsis lyrata subsp. lyrata]|XP_020870018|	germin-like protein subfamily T member 1 [Arabidopsis lyrata subsp. lyrata]|XP_020870018|
pathogenesis-related 4 [Arabidopsis thaliana] |NP_187123|		pathogenesis-related 4 [Arabidopsis thaliana] |NP_187123|
	LOW QUALITY PROTEIN: cytosolic isocitrate dehydrogenase [NADP] [Arabidopsis lyrata subsp. lyrata]|XP_020891091|	
	metallothionein 3 [Arabidopsis thaliana] |NP_566509|	*metallothionein 3 [Arabidopsis thaliana] |NP_566509|*
	Peroxidase superfamily protein [Arabidopsis thaliana] |NP_196153|	
pectinesterase inhibitor 12 [Arabidopsis lyrata subsp. lyrata] |XP_002863381|		pectinesterase inhibitor 12 [Arabidopsis lyrata subsp. lyrata] |XP_002863381|
plasma membrane intrinsic protein 1C [Arabidopsis thaliana] |NP_001077441|		plasma membrane intrinsic protein 1C [Arabidopsis thaliana] |NP_001077441|

PLAT/LH2 domain-containing lipoxygenase family protein [Arabidopsis thaliana] |NP_177396|	PLAT/LH2 domain-containing lipoxygenase family protein [Arabidopsis thaliana] |NP_177396|	PLAT/LH2 domain-containing lipoxygenase family protein [Arabidopsis thaliana] |NP_177396|
putative 1,4-alpha-glucan branching enzyme protein soform SBE2.2 [Arabidopsis thaliana]|BAC42378|	putative 1,4-alpha-glucan branching enzyme protein soform SBE2.2 [Arabidopsis thaliana]|BAC42378|	
RmlC-like cupins superfamily protein [Arabidopsis thaliana] |NP_173332|	RmlC-like cupins superfamily protein [Arabidopsis thaliana] |NP_173332|	RmlC-like cupins superfamily protein [Arabidopsis thaliana] |NP_173332|
putative AP2 domain transcriptional regulator, 5' partial; 1-558, partial [Arabidopsis thaliana]|AAG52091|		putative AP2 domain transcriptional regulator, 5' partial; 1-558, partial [Arabidopsis thaliana]|AAG52091|
putative esterase [Arabidopsis thaliana]|AAM61628|		putative esterase [Arabidopsis thaliana]|AAM61628|
putative L-asparaginase [Arabidopsis thaliana]|BAC42877|		putative L-asparaginase [Arabidopsis thaliana]|BAC42877|
putative non-LTR retroelement reverse transcriptase [Arabidopsis thaliana]		putative non-LTR retroelement reverse transcriptase [Arabidopsis thaliana]
S-adenosyl-L-methionine-dependent methyltransferases superfamily protein [Arabidopsis thaliana] |NP_195131|	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein [Arabidopsis thaliana] |NP_195131|	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein [Arabidopsis thaliana] |NP_195131|
stem-specific protein TSJT1 [Arabidopsis lyrata subsp. lyrata] |XP_002894413|	stem-specific protein TSJT1 [Arabidopsis lyrata subsp. lyrata] |XP_002894413|	
sulfurtransferase 18 [Arabidopsis thaliana] |NP_001190631|		sulfurtransferase 18 [Arabidopsis thaliana] |NP_001190631|
zinc ion-binding protein [Arabidopsis thaliana] |NP_563644|		

Table 2. Effects of 15% PEG with or without 7 days of 0.5 mM putrescine pre-treatment on the glutathione reductase (GR), ascorbate peroxidase (APX) and guaiacol peroxidase (POD) enzyme activities (nkatal g^{-1} FW) after 5 days in wheat plants. Data represent mean values ±SD, n=5. Different letters indicate significant differences between the treatments at $P< 0.05$.

Enzymes	control		PUT pre-treated		PEG		PUT pre-treated+PEG	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
GR	22.87±3.21 ab	4.89±0.42 a	23.34±1.21 a	4.95±.0.5 a	25.73±0.89 ab	7.67±0.06 b	27.56±1.6 b	9.84±1.8 b
APX	145.82±14.8 ab	185.95±13.0 a	145.39±7.75 a	211.5±16.19 a	168.32±9.31 b	223.61±20.98 ab	169.13±15.3 ab	277.23±35.44 b
POD	234.29±26.84 a	697.95±87.47 a	257.05±34.6 ab	896.24±109.72 ab	309.86±23.68 b	887.76±47.62 b	277.77±34.09 ab	1183.22±101.35 c

Table 3. Effects of 15% PEG with or without 7 days of 0.5 mM putrescine pre-treatment on the on the fatty acid composition (mol%), double bond index (DBI) and percentage of unsaturation (%unsat) of various lipid classes obtained from wheat roots. Data represent mean values ± SD (n=3); different letters denote significant differences from control samples taken on the same day at $p < 0.05$.

Fraction	Treatment	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	DBI	%unsat
MGDG	**C**	1.52±0.26 a	nd	0.38±0.17 a	0.35±0.02 a	2.31±0.39 ab	95.44±0.83 a	291.31±1.70 b	98.10±0.42 b
	PUT	**2.97±0.07 b**	nd	**1.41±0.25 b**	0.61±0.15 a	2.66±0.3 ab	92.60±1.13 a	283.73±2.63 ab	**95.63±0.34 a**
	PEG	2.28±0.03 a	nd	**1.20±0.07 b**	1.00±0.38 a	2.46±0.06 a	93.05±0.36 a	**285.08±0.80 a**	**96.51±0.04 a**
	PUT+PEG	2.11±0.31 ab	nd	0.82±0.09 ab	0.55±0.2 a	**2.99±1.14 b**	93.52±0.47 a	287.09±1.49 ab	97.06±0.41 ab
DGDG	**C**	8.96±0.06 a	nd	1.21±0.08 a	1.33±0.18 a	2.46±0.71 a	86.64±1-88 ab	265.56±3.20 ab	89.83±0.14 c
	PUT	9.88±0.01 b	nd	1.39±0.27 ab	1.04±0.21 a	2.6±0.14 a	85.10±0.44 b	261.53±1.07 b	**88.74±0.26 b**
	PEG	9.73±0.07 b	nd	1.76±0.04 b	1.21±0.13 a	2.11±0.49 a	85.18±0.60 b	260.98±0.67 b	**88.51±0-03 b**
	PUT+PEG	11.06±0.06 c	nd	2.43±0.14 c	1.52±0.18 a	2.55±0.10 a	82.43±0.01 a	253.91±0.003 a	**86.50±0.09 a**
PE	**C**	44.44±8.00 a	nd	22.58±3.12 a	10.78±0.32 b	13.34±1.72 a	15.56±3.06 a	84.15±6.05 a	37.19±4.88 a
	PUT	42.90±9.53 a	nd	27.81±6.45 a	12.31±1.93 ab	14.96±0.13 a	15.16±0.55 a	87.72±3.84 a	37.83±3.91 a
	PEG	31.00±0.68 a	nd	16.93±0.23 a	11.41±1.78 ab	15.87±0.93 a	**24.79±1.80 b**	**117.54±1.78 b**	**52.08±0.90 b**
	PUT+PEG	29.10±1.23 a	nd	17.37±0.71a	8.00±0.76 a	16.05±0.37 a	**29.47±2-32 b**	**128.53±6.95 b**	**53.53±1.94 b**
PG	**C**	18.85±0.46 a	28.51±1.06 a	4.09±0.45 a	2.19±0.16 a	6.97±0.10 b	39.39±1.15 ab	134.30±3.47 ab	48.55±1.08 ab
	PUT	19.92±1.39 a	26.33±1.43 a	5.03±0.60 a	1.86±1.03 ab	**6.10±0.06 a**	40.75±2.45 ab	136.32±8.26 ab	48.71±3.42 ab
	PEG	**21.03±0.33 b**	26.46±0.81 a	5.06±0.55 a	2.60±0.27 a	7.20±0.22 b	37.65±0.09 a	129.96±0.99 a	47.45±0.58 a
	PUT+PEG	19.93±0.62 ab	25.46±0.23 a	4.55±0.23 a	3.76±0.25 a	**6.00±0.13 a**	40.29±0.25 b	136.64±1.25 b	50.05±0.62 b

MGDG: monogalactosyldiacylglycerol ; DGDG: digalactosyldiacylglycerol, PE: phosphatidylethanolamine and PG: phosphatidylglycerol; *t*16:1: *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. nd: not detected.

Captions

Figure 1. A Scatter plot diagram of the expression datasets derived from principal component analysis based on the logFC values of the genes. All genes were plotted with respect to the first and second principal components (PC1, PC2), and indicated by blue text. The axes represent PC1 and PC2. The biplot representation (green lines) shows the projection of the original variables (light regimes) onto the scatter diagram. **B** The loadings of the original variables along the PC1.

Figure 2. Multi set Venn diagrams show the number of up- (A) and downregulated (B) genes in the overlaps and those expressed individually after an $e<1^{-4}$ cut-off.

Figure 3. Gene ontology (GO) analysis of C vs. PEG, C vs. PUT, C vs. PUT+PEG, PEG vs. PUT, PUT vs. PUT+PEG and PEG vs. PUT+PEG comparisons was carried out on *Arabidopsis* protein accessions. Overrepresented terms in the classes 'biological process' (A), 'molecular functions' (B) and and 'cellular component' (C) are shown in colored boxes ($q<0.05$; q-value means the multiple-test adjusted p-value). Hierarchical graphs show the level of significance of the terms and the linkage among them. Box colors indicate levels of statistical significance: white = parent or non-significant term; yellow $= 0.05$ and brown $= e^{-5}$. Arrows mean conjunctions: solid = is a; dashed = two significant nodes; dotted = one significant node and green = negative regulate. The pair of numerals in the left represents number of genes in query list associated with that GO term and number of genes in query list. The pair of numerals in the right represents number of genes associated with a particular GO term in the TAIR10 database and total number of *Arabidopsis* genes with GO annotations in the TAIR10 database.

Figure 4. Guaiacol peroxidase isoenzymes (PODs) after 15% PEG with or without 7 days of 0.5 mM putrescine pre-treatment in the leaves (A: LPODs) and roots (B: RPODs) of wheat plants. Proteins were separated in 10 % non-denaturing polyacrylamide gels. Arrows indicate peroxidase isoenzyme bands. Equal amounts of crude extract were loaded on the gel in case of

each treatment. Lanes labelled with odd-numbered lines were loaded by four times as much amount of sample as the even-numbered ones.

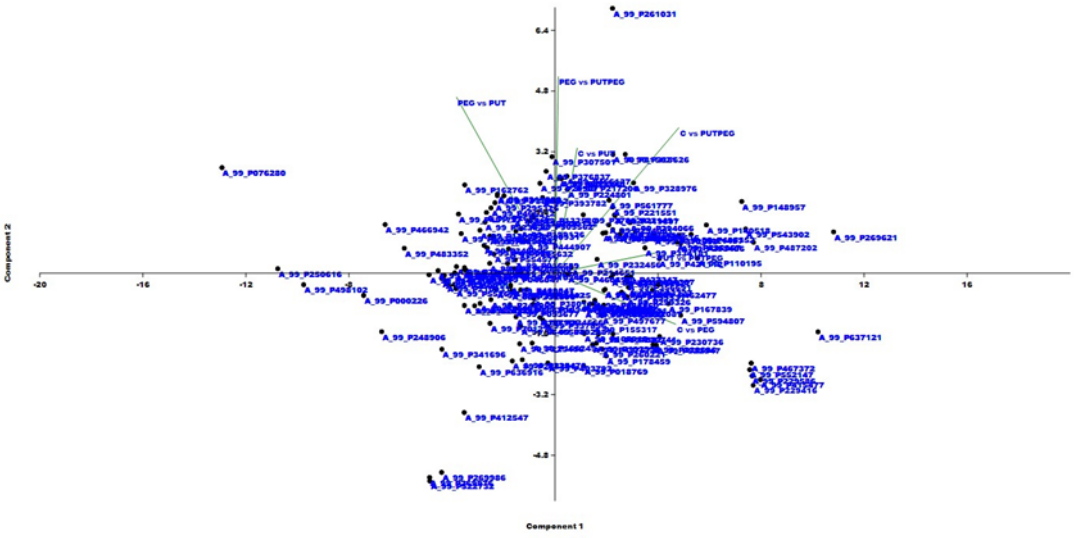
Suppl. Figure 1. Validation of the microarray results. Microarray data (least-square means) were plotted against data from qRT-PCR and fitted into a linear regression. Both the x- and y-axes are shown on a log 2 scale.

Suppl. Figure 2. Validation of the microarray data.

Suppl. Table 1. List of array probes significantly differentially expressed (logFC > 2 and p< 0.05) of C vs. PEG, C vs. PUT, C vs. PUT+PEG, PEG vs. PUT, PUT vs. PUT+PEG and PEG vs. PUT+PEG with the following attributes: probe name, corrected p-value, p-value, probe sequence, EST accession in *Triticeae* taxon, refseq protein ID in *Triticeae* taxon, description in *Triticeae* taxon, expectation (e) value in *Triticeae* taxon, refseq protein ID in *Arabideae* taxon, description in *Arabideae* taxon, expectation (e) value in *Arabideae* taxon, expression, fold-change, log-fold-change and absolute fold-change.

Suppl. Table 2. Lists of gene functions of wheat derived from the Venn diagram of C vs PEG, C vs PUT, C vs PUT+PEG, PEG vs PUT, PUT vs PUT+PEG and PEG vs PUT+PEG comparisons and their overlaps which contain gene function. The up- and down-regulated lists are presented on separate sheets.

A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PEG vs PUTPEG
PEG vs PUT
C vs PUTPEG
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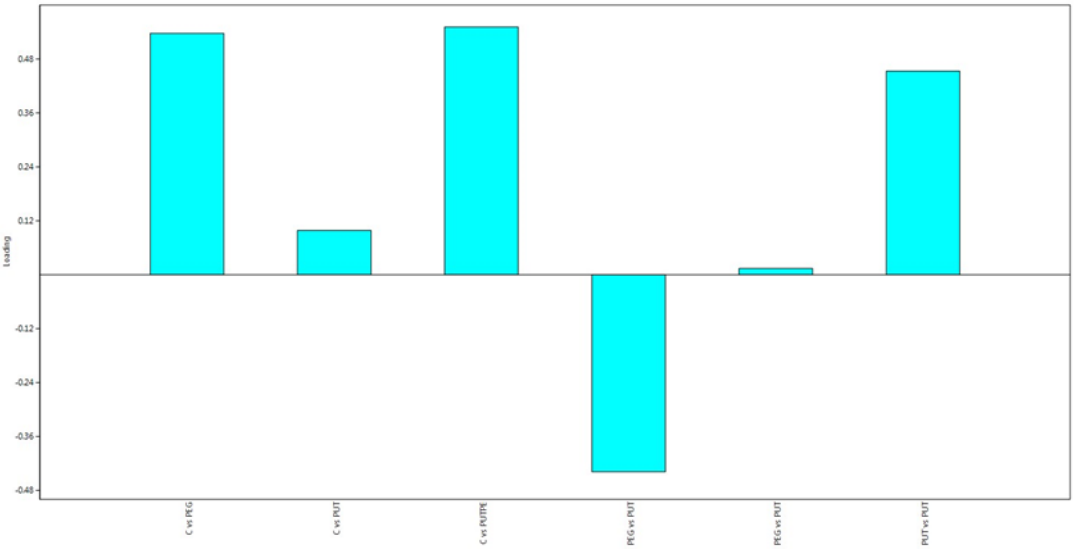
Component 1
Component 2

B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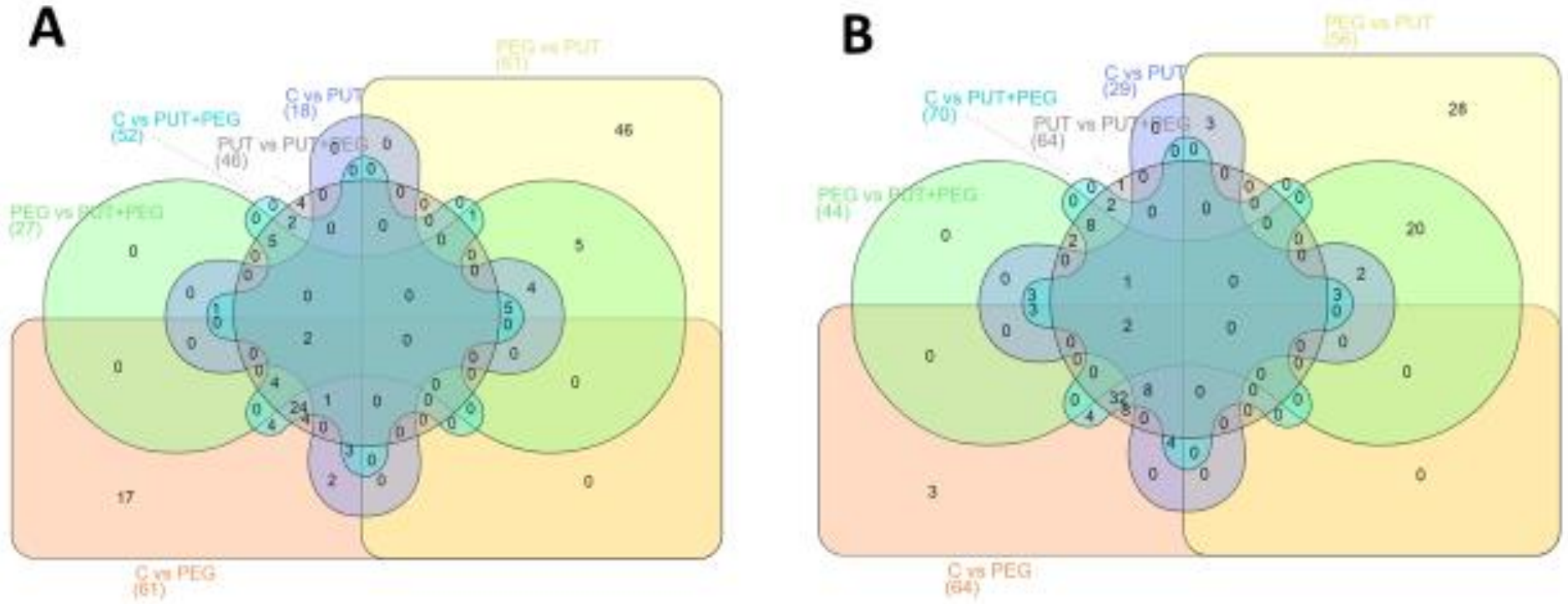
C vs PUT
C vs PUTPE
PEG vs PUT
PEG vs PUT
PUT vs PUT


A
C vs PUT
(18)
C vs PUT+PEG
(52)
PUT vs PUT+PEG
(48)
PEG vs PUT
(61)
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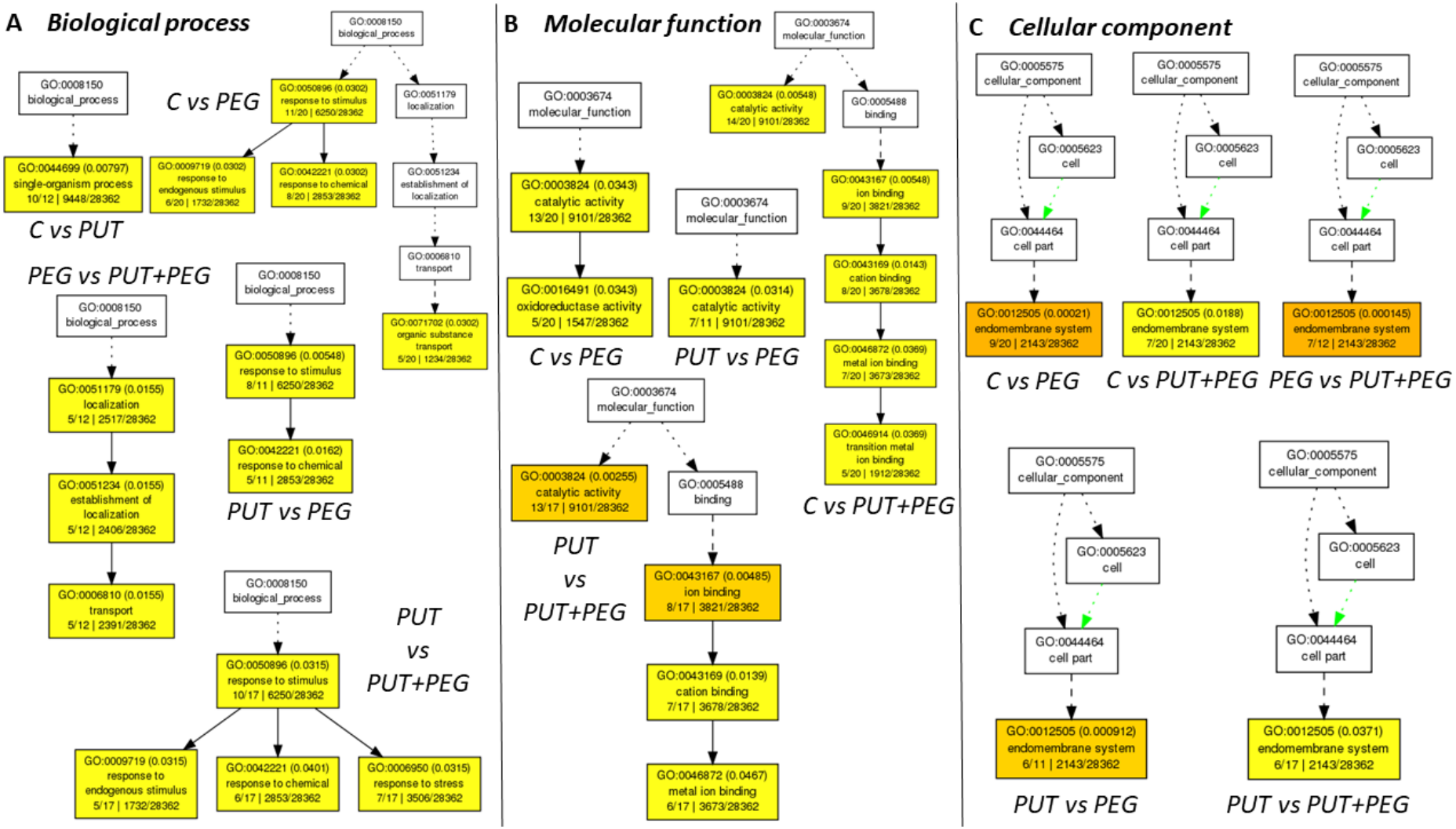
(27)
C vs PEG
(61)
B
C vs PUT
(29)
C vs PUT+PEG
(70)
PUT vs PUT+PEG
(64)
PEG vs PUT
(56)
PEG vs PUT+PEG
(44)
C vs PEG
(64)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B Molecular function
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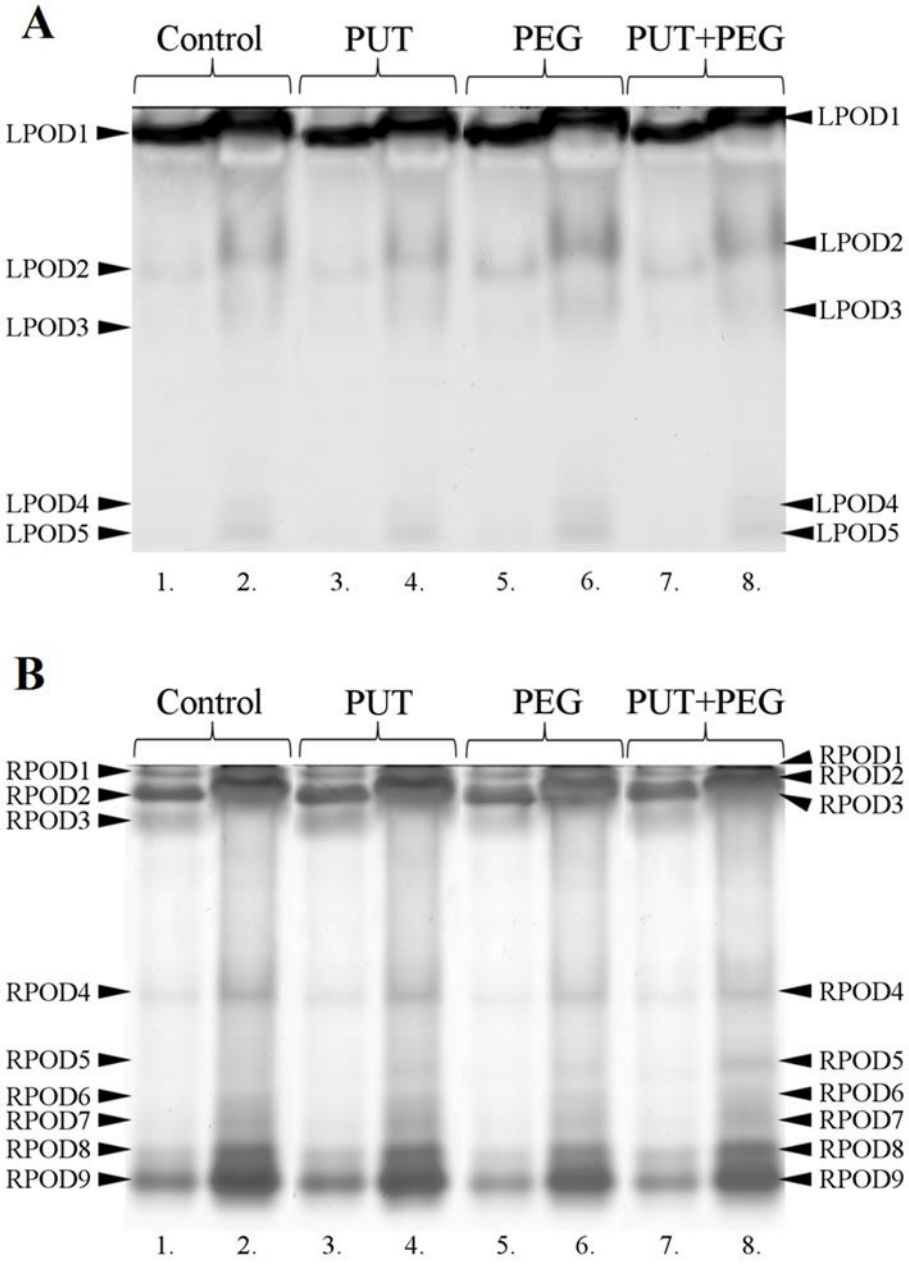
PUT vs PUT+PEG
C Cellular component
C vs PEG
C vs PUT+PEG
PEG vs PUT+PEG
PUT vs PEG
PUT vs PUT+PEG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PEG
PUT+PEG
LPOD1
LPOD2
LPOD3
LPOD4
LPOD5
1. 2. 3. 4. 5. 6. 7. 8.
B
Control
PUT
PEG
PUT+PEG
RPOD1
RPOD2
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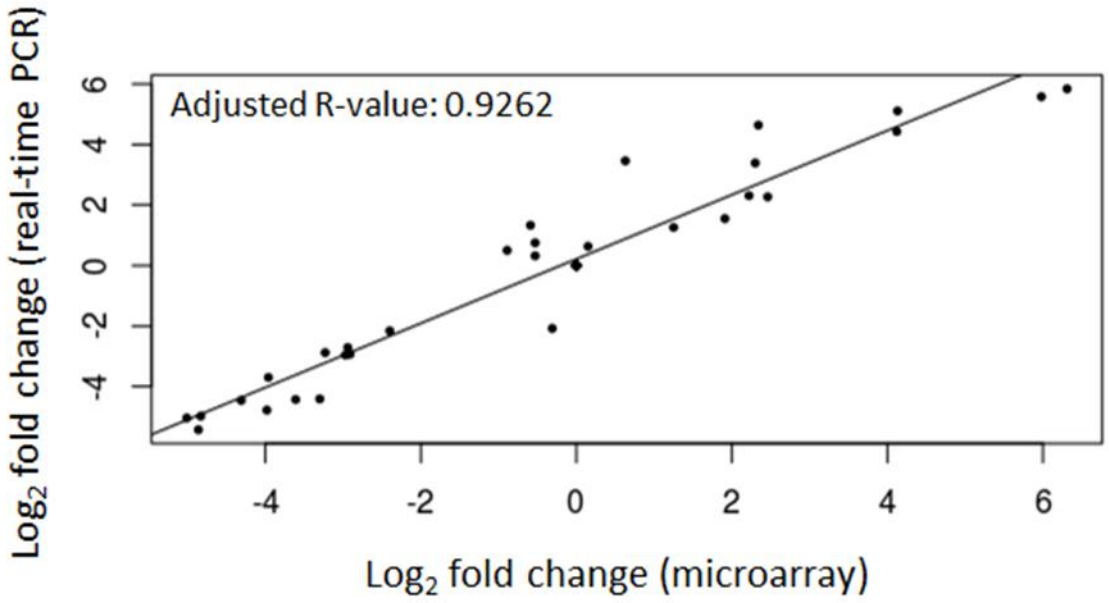
RPOD4
RPOD5
RPOD6
RPOD7
RPOD8
RPOD9
1. 2. 3. 4. 5. 6. 7. 8.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Log2 fold change (microarray)
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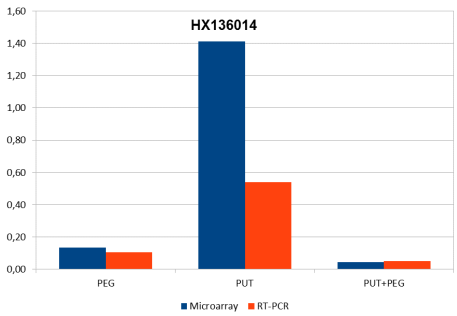
2
4
6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PUT
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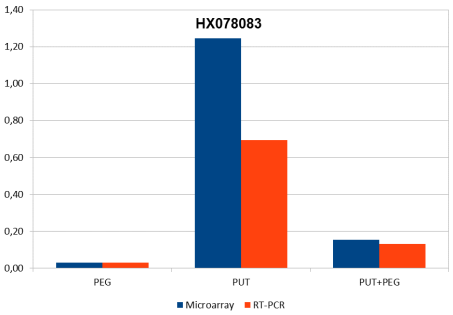
Microarray
RT-PCR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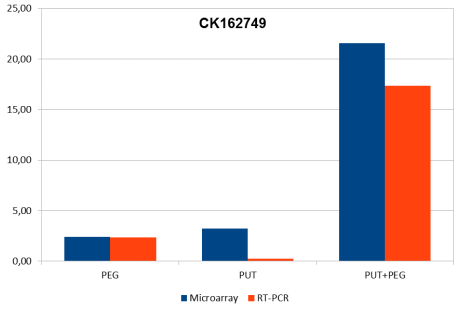
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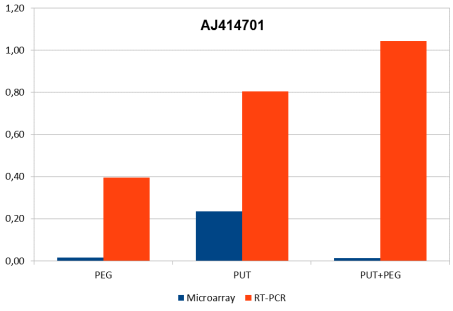
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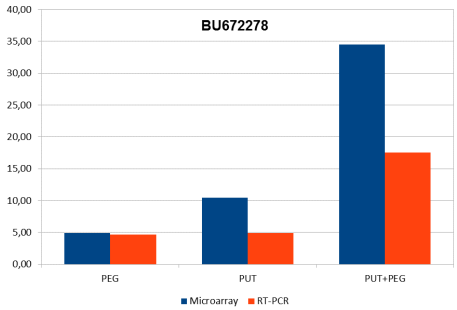
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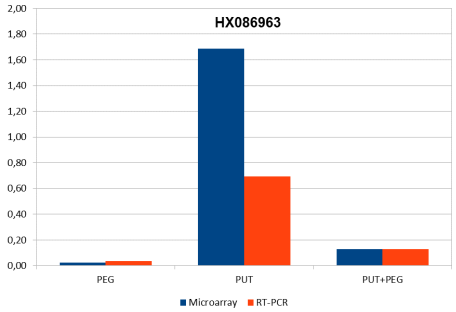
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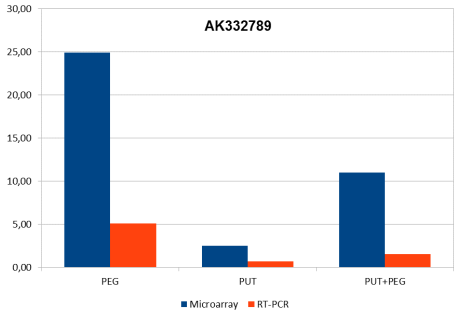
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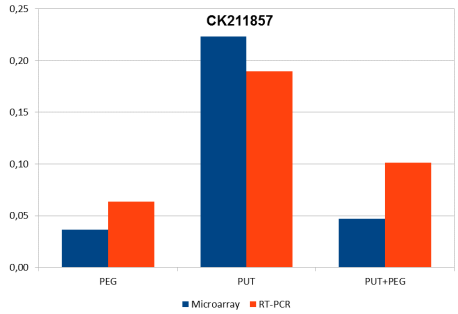
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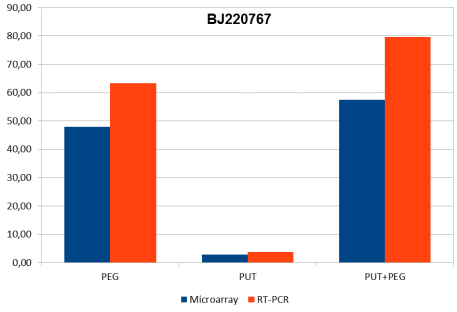
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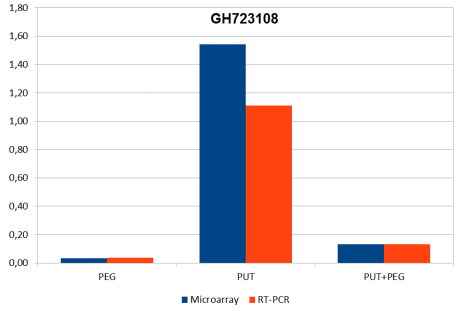
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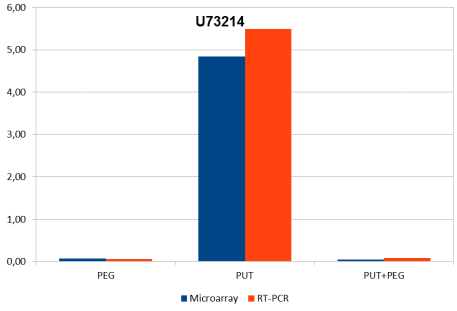
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