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RESEARCH PAPER

Changes in the effective gravitational field strength affect the state of phosphorylation of stress-related proteins in callus cultures of *Arabidopsis thaliana*

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Abstract

In a recent study it was shown that callus cell cultures of *Arabidopsis thaliana* respond to changes in gravitational field strengths by changes in protein expression. Using ESI-MS/MS for proteins with differential abundance after separation by 2D-PAGE, 28 spots which changed reproducibly and significantly in amount (P <0.05) after 2 h of hypergravity (18 up-regulated, 10 down-regulated) could be identified. The corresponding proteins were largely involved in stress responses, including the detoxification of reactive oxygen species (ROS). In the present study, these investigations are extended to phosphorylated proteins. For this purpose, callus cell cultures of *Arabidopsis thaliana* were exposed to hypergravity (8 *g*) and simulated weightlessness (random positioning; RP) for up to 30 min, a period of time which yielded the most reliable data. The first changes, however, were visible as early as 10 min after the start of treatment. In comparison to 1 *g* controls, exposure to hypergravity resulted in 18 protein spots, and random positioning in 25, respectively, with increased/decreased signal intensity by at least 2-fold (P <0.05). Only one spot (alanine aminotransferase) responded the same way under both treatments. After 30 min of RP, four spots appeared, which could not be detected in control samples. Among the protein spots altered in phosphorylation, it was possible to identify 24 from those responding to random positioning and 12 which responded to 8 *g*. These 12 proteins (8 *g*) are partly (5 out of 12) the same as those changed in expression after exposure to 2 h of hypergravity. The respective proteins are involved in scavenging and detoxification of ROS (32%), primary metabolism (20.5%), general signalling (14.7%), protein translation and proteolysis (14.7%), and ion homeostasis (8.8%). Together with our recent data on protein expression, it is assumed that changes in gravitational fields induce the production of ROS. Our data further indicate that responses toward RP are more by post-translational protein modulation (most changes in the degree of phosphorylation occur under RP-treatment) than by protein expression (hypergravity).

Key words: *Arabidopsis thaliana*, cell cultures, hypergravity, phosphoproteomics, ROS detoxification, simulated microgravity.

Introduction

Gravitation has a profound influence on plant development and orientation in space. Recent studies have shown that exposure of *Arabidopsis* seedlings and callus cells to altered gravitational forces (clinorotation or hypergravity) induces changes in gene expression (Moseyko *et al.*, 2002; Centis-Aubay *et al.*, 2003; Martzivanou and Hampp, 2003; Yoshioka *et al.*, 2003; Kimbrough *et al.*, 2004; Babbick *et al.*, 2007). Changes in transcript levels are, however,

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largely treatment-specific. While some transcripts were not affected by any treatment, some were altered in an opposite manner by clinostat and hypergravity conditions, or desensitized to hypergravity after extended clinorotation, and vice versa (Centis-Aubay *et al.*, 2003). Callus cultures derived from stems of *A. thaliana* seedlings responded to hypergravity (between 2 *g* and 10 *g*) by an up-regulated expression of approximately 200 genes (Martzivanou and Hampp, 2003). Twelve percent of the respective transcripts were involved in cellular signalling as well as protein phosphorylation and dephosphorylation, 6% in defence and stress response, and 2% in gravisensing. Although changes in transcript levels are not necessarily related to the expression of the corresponding proteins (owing to proteolytic processing, post-translational modifications etc.) such changes should, with some delay in time, also affect protein expression patterns. Accordingly, recent studies showed that both clinorotation and hypergravity alter protein patterns as obtained by 2D-PAGE of soluble proteins (Barjaktarovic *et al.*, 2007, 2008; Wang *et al.*, 2006). Identification of some spots indicated that these proteins were involved in carbohydrate and lipid metabolism, signalling, gene expression, and cell wall biosynthesis (Wang *et al.*, 2006). In a recent proteomic study where responses to hypergravity, clinoroation, and random positioning were compared, significant changes were detected in the amounts of individual protein spots after just 2 h of exposure to 8 *g* (Barjaktarovic *et al.*, 2007). Separation of soluble proteins by 2D-PAGE and subsequent identification of the resolved spots by mass spectrometry (*nano*-HPLC-ESI-MS/MS; high-pressure liquid chromatography–electrospray ionization tandem mass spectrometry) revealed 28 specific proteins. According to their metabolic function, it is assumed that hypergravity may cause some kind of oxidative stress.

As our interest was mainly in short-term responses to changes in the effective gravitational field, these studies were extended to phosphorylated proteins.

Protein phosphorylation/dephosphorylation is one of the first events in signal transduction cascades and is a way of regulation of many cellular functions (for a review see Huber, 2007). Most recent investigations have identified more than 5000 protein phosphorylation sites (Olsen *et al.*, 2006; Villén *et al.*, 2007). In the genome of *Arabidopsis* about 1100 protein kinases and more than 100 protein phosphatases exist (Kerk *et al.*, 2002; The *Arabidopsis* Genome Initiative, 2000). Phosphorylation of a specific protein leads to the activation/deactivation of its function or can serve for protein assembly and interactions with other proteins such as, for example, 14-3-3 regulatory proteins. Although phosphorylated proteins can represent up to 30% of the total protein population, their separation and enrichment has been challenging in the past. Recently, several phosphoproteomic studies have been successfully performed by direct phosphoprotein staining of one and two dimensional protein gels (Schulenberg *et al.*, 2003; Chitteti and Peng, 2007; Eymann *et al.*, 2007; Tan *et al.*, 2007). Using the latter approach, it is shown here that gravitation-related alterations in the degree of protein phosphorylation can be detected as early as 10 min after exposure to hypergravity or random positioning, with maximum significant phosphorylation events after 30 min. Spot analysis by *nano*-HPLC-ESI-MS/MS revealed changes in the degree of phosphorylation for several proteins which are responsive to reactive oxygen species. This is in support of our earlier findings which indicate that gravitation-sensing obviously involves signals related to oxidative stress (Barjaktarovic *et al.*, 2007).

Materials and methods

Cell cultures

Cell suspension cultures were generated from leaves of *Arabidopsis thaliana* (cv. Columbia) plants, grown under sterile conditions. Calli obtained on 1.6% agar plates containing I.2a medium [80 mM sucrose, Murashige and Skoog basal salt medium (4.4 g l^{-1}; Sigma), mixture of amino acids and phytohormones according to Seitz and Alfermann, 1985] were transferred to a liquid I.2a medium (200 ml without agar in 500 ml Erlenmeyer flasks) and grown at 26 °C in the dark on a rotary shaker (Infors, Bottmingen, Switzerland; 130 rpm). New medium was added every week. This suspension culture was taken as stock for repeated callus formation. For this purpose, cell suspensions (10 ml) were spread on 9 cm plates containing agar/I.2a medium as above, with surplus medium being decanted. Calli with a diameter of about 1 mm were obtained after 1 week of growth and used for the experiments. At the end of exposure, calli were rapidly scraped off the agar (less than 5 s), submerged in liquid nitrogen, and stored at –80 °C until use. For each time point the corresponding control samples were collected, which were in close proximity to the exposed samples (inside the centrifuge housing; in the temperature-controlled RPM room).

Application of hypergravity (8 g) and simulation of microgravity by random positioning

The application of 8 *g* was by centrifugation of the Petri dishes. The radius (distance between the centre of centrifuge axis and the centre of the Petri dish) was determined to adjust the corresponding rpm number. Petri dishes were fixed in holders, the angle of which could be adjusted by micrometer screws. The angle was set such that the resulting centrifugal force was applied perpendicular to the Petri dish surface.

For random positioning, cultures were prepared on Petri dishes as described above for centrifugation. The dishes were then fixed in the centre of the inner of the two connected frames. The frames were rotating in a random, autonomous way at a maximal angular velocity of 60° s^{-1} (Walther *et al.*, 1998). For details of the different procedures, see Babbick *et al.* (2007).

Protein extraction

The extraction procedure is based on the method of Niini *et al.* (1996) with some modifications. One gram fresh

weight of *Arabidopsis* calli was ground to a fine powder in liquid nitrogen and transferred to 2 ml of lysis buffer (40 mM TRIS/HCl, pH 8, containing both a plant protease inhibitor cocktail and a phosphatase inhibitor cocktail II according to the manufacturers suggestions (20 µl each; Sigma-Aldrich, Munich, Germany). The suspensions were kept on ice for 30 min with gentle vortexing every 10 min. Then, 2.5 ml water-saturated phenol was added, the samples were subjected to a freeze (liquid nitrogen) thaw cycle, and shaken at 4 °C for 1 h. After centrifugation at 10 000 rpm for 10 min, the phenol phase was washed twice with 2 ml of lysis buffer (see above). Proteins were precipitated with 3 vols of 10% TCA for 1 h at –20 °C and centrifuged at 15 000 rpm for 2 h. The pellet was washed twice with 1 ml acetone, and resolubilized in a buffer containing 9.5 M urea (increased solubilization), 60 mM DTT, 2% CHAPS and 0.5% ampholines, pH 3–10 (GE Healthcare, Munich, Germany). The protein concentration was determined using the Bradford assay (Bio-Rad, Munich, Germany) with BSA as standard.

2-D gel electrophoresis

IEF was performed using immobilized pH gradients (18 cm; pH range 4–7; GE Healthcare, Munich, Germany). 250 µg of protein in rehydration buffer (8 M urea, 2% CHAPS, 30 mM DTT, 0.5% ampholines, pH 4–7) were loaded onto the gel strips in a Protean IEF cell (Bio-Rad, Munich, Germany) Isoelectric focusing was carried out at 20 °C, using voltages and running times as follows: 12 h passive rehydration, rapid 300 V for 1 h, gradients from 300 to 1000 V for 2 h, 1000 to 3500 V for 2 h, 3500 to 8000 V for 2 h, rapid 8000 V for 1 h, gradient 8000 to 10 000 V for 1 h, and finally rapid 10 000 V up to a total of ~65 kVh). The maximum current was 50 µA per gel strip. Gel strips were incubated in equilibration solution (50 mM TRIS/HCl, pH 8.8, 6 M urea, 2% (w/v) SDS, 1% (w/v) DTT) for 15 min, followed by a second 15 min incubation with the same solution, but DTT was substituted by 2.5% (w/v) iodoacetamide). Equilibrated gel strips were placed on top of a 12% acrylamide gel (PDA as a crosslinker, Bio-Rad, Munich, Germany) and overlaid with 0.5% agarose solution. SDS-PAGE was carried out using the large Protean II cell (Bio-Rad), and performed at 20 mA for 1 h, followed by 40 mA for 5 h.

Staining procedure, image scanning, and image analysis

The 2D gels were stained with Pro-Q Diamond (Invitrogen, Karlsruhe, Germany) according to a modified protocol of Agrawal and Thelen (2005). After electrophoresis, gels were fixed twice in 50% methanol and 10% acetic acid, first for 30 min and then overnight. After washing in double distilled water four times for 15 min, gels were stained with 3-fold diluted Pro-Q Diamond for 2 h. To remove non-specific background staining, gels were destained three times for 30 min in 20% acetonitrile and 50 mM sodium acetate, pH 4. Prior to image scanning, the gels were washed twice in double distilled water. Image scanning was performed with a FLA-5100 instrument (Fuji, Japan), using the 532 nm laser source and a 580 nm emission filter. The same gels were then stained using a silver staining protocol for total protein detection (Blum *et al.*, 1987). Briefly, gels were sensitized for 30 min in 30% (v/v) ethanol, 8 mM sodium thiosulphate and 500 mM sodium acetate, washed three times for 5 min in water, stained with 30 mM silver nitrate, developed with 235 mM sodium carbonate, containing 1/2000 vol. of 37% formaldehyde and 1/4000 vol. of 10% sodium thiosulphate solution. The reaction was stopped by the addition of 200 ml 0.5% (w/v) glycine. The gels were scanned in a Fuji FLA 5100. Image analysis and spot quantification was performed using PD QUEST software 8.01 (Bio-Rad, Munich, Germany). Protein spots were assumed to be differentially phosphorylated when (a) the spot quantity was changed in intensity by at least 2-fold with a significance level of 95% according to Student *t* test, or (b) appeared or disappeared in three independent replicates. In order to be able to detect the respective spots after staining for total protein, the Pro-Q Diamond-stained gel was aligned with the corresponding silver-stained gel by means of the software Delta 2D (version 3.6, DECODON GmbH, Greifswald, Germany), and using 17 landmarks (Fig. 1). After the alignment of gels, the boundaries of spots of interest were then transferred from the Pro-Q to the silver-stained gels. This way the relevant spots were located, and then cut out for identification by mass spectrometry.

Mass spectrometry and protein identification

Spots were excised manually from the gel and proteins were digested overnight with trypsin (sequencing grade,

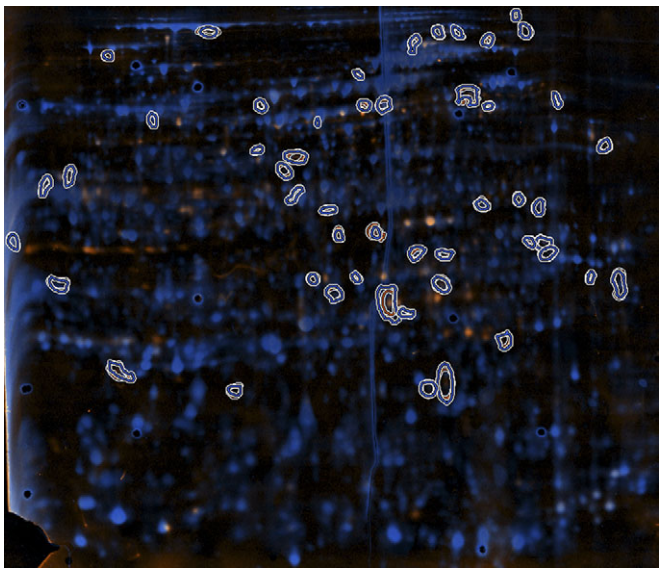

Fig. 1. Dual channel image of total protein (silver-staining: blue) and Pro-Q Diamond-staining (red). The Pro-Q Diamond-stained gel was aligned with the corresponding silver-stained gel by means of the software Delta 2D, using 17 landmarks.

Promega, Mannheim, Germany). Eluted peptides were analysed using a Dionex LC Packings nano-HPLC system (Idstein, Germany) coupled to an electrospray mass spectrometer (QStar Pulsar i, Applied Biosystems, Darmstadt, Germany). The ESI-MS/MS spectra were recorded as detailed elsewhere (Hála *et al.*, 2008). Proteins were identified by correlating the ESI-MS/MS spectra with the NCBI.nr-protein sequence database of *Arabidopsis thaliana* as of 8 June 2007, using the MOWSE-algorithm as implemented in the MS search engine MASCOT (Matrix Science Ltd., London, UK; Perkins *et al.*, 1999). All experimental data, achieved by 2D gel electrophoresis and mass spectrometry, as well as corresponding search results, were stored in a LIMS database (Proteinscape 1.3, Bruker Daltonics, Bremen, Germany).

Results

Figure 2A and B gives examples for 2D-PAGE separations of phosphorylated proteins after staining with Pro-Q Diamond. In comparison to the 1 *g* controls, exposure to hypergravity resulted in 18 protein spots, and random positioning in 25 spots, respectively, with increased/decreased signal intensity (PD Quest) by at least 2-fold (P <0.05). After 30 min RP, four spots appeared, which could not be detected in control samples. From the spots that had altered in amount, it was possible to identify 24 proteins from those responding to random positioning and 12 which responded to 8 *g*. The remaining spots could not be identified due to the very low amount of the corresponding protein on the gels.

Tables 1 and 2 contain the respective protein identifications according to their highest homologies with fragment pattern etc., reported for known proteins from *A. thaliana*. According to function, the proteins altered in their degree of phosphorylation are involved in ROS (reactive oxygen species) scavenging and detoxification (32%), primary metabolism (20.5%), general signalling (14.7%), protein translation and proteolysis (14.7%), and ion homeostasis (8.8%). The predicted and experimental phosphorylation sites and motifs that bind to domains such as 14-3-3 proteins or protein kinases were checked using ELM (http://elm.eu.org), Scansite (http://scansite.mit.edu), and NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/).

Discussion

For most of the proteins which change their degree of phosphorylation it is not yet known how this affects their activity or their interaction with other proteins. Most of the changes found show an increase in the amount of the phosphorylated protein in relation to the 1 *g* control (24). For proteins such as aldehyde dehydrogenase, CPN60B (CPN60), CPN60A (HSP60), or alanine aminotransferase (30 min, 8 *g*) this should mean increased activity, because these proteins also appeared to be increased in amount after 2 h of exposure to hypergravity (Barjaktarovic *et al.*, 2007). But there are also quite a few (12) with a decrease in phosophorylation. In the case of enolase, it is known that this enzyme is activated by dephosphorylation (see below). Thus, in this case a decrease in the degree of phosphorylation means an increase in the rate of activity.

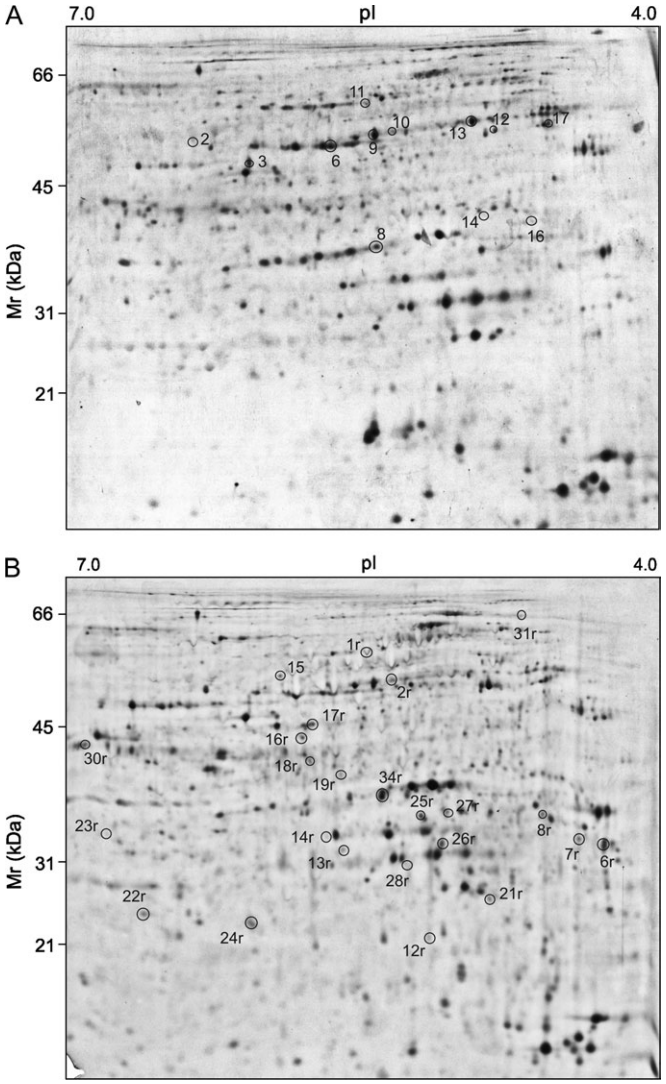

Fig. 2. Two-dimensional separation of phosphoproteins from *Arabidopsis* callus cells. The gels were stained with Pro-Q Diamond. Indicated spots are those with increased/decreased signal intensity (PD Quest) by at least 2-fold (P <0.05) after 30 min of 8 *g* (A) and 30 min of random positioning treatment (B). The ranges of pI and molecular masses are indicated.

In the following, the functions of the identified proteins are discussed as groups.

ROS scavenging and detoxifying enzymes

Extracellular signals such as those resulting from biotic or abiotic stresses are recognized by membrane-located receptors or sensors and are translated into the intracellular formation of signal mediators. Reactive oxygen species form an important group of such intermediates. Biotic as well as abiotic stresses induce a reduction of molecular oxygen by membrane-bound NADPH oxidase, giving rise

Table 1. Identification of proteins from *Arabidopsis thaliana* callus cells that are differentially phosphorylated (>2-fold up or down, $P < 0.05$) after 30 min of exposure to random positioning

Spots marked with numbers 18r, 21r, 8r, and 28r appeared after treatment and could not be detected in 1 *g* controls. MW, molecular mass of predicted proteins; pI, pI value of predicted proteins; Sequence coverage (%), percentage of predicted protein sequence covered by matched peptides; No. matched, number of peptides matched. NCBI no., accession number; MASCOT score, probability-based MOWSE score: $-10\times\text{Log}(P)$, where P is the probability that the observed match is a random event. Scores greater than 34 indicate identity or extensive homology; $P < 0.05$.

Spot no.	NCBI no.	*Arabidopsis* protein name	MW(kDa)	pI	Sequence coverage	Mascot score	No matched peptides	Ratio RP/ control
ROS related								
15r	NP_565954	Thioredoxin reductase	57.9	6.31	11	269	7	0.48
17r	NP_188929	ATP sulphurylase 3	51.4	6.34	21	554	12	3.55
31r	NP_191056	Protein disulphide isomerase	64.2	4.74	6	117	4	6.08
23r	NP_195226	Ascorbate peroxidase 3	31.6	6.47	21	237	7	4.41
12r	NP_180715	Glutathione peroxidase	18.9	5.6	39	243	7	2.66
7r	BAD95030	Heat shock protein 83	27.8	4.46	7	84	2	2.73
6r	CAC16575	p23 co-chaperone	25.4	4.44	19	171	6	0.31
19r	NP_177837	Alcohol dehydrogenase	41.2	5.83	12	252	5	2.19
Metabolism								
13r	BAB11233	6-phosphogluconolactonase	28	5.44	17	335	6	2.6
28r	NP_191104	Triose phosphate isomerase	27.2	5.39	28	392	6	appear
30r	NP_181187	Fructose-bisphosphate aldolase	38.4	7.01	33	676	14	0.38
1r	AAB60731	Phosphoglycerate mutase	62.6	5.36	24	895	17	4.07
2r	AAF79891	Alanine aminotransferase	51.7	5.39	17	396	9	0.5
General signalling								
8r	NP_566174	14-3-3 like protein	29.8	4.74	18	245	6	appear
24r	NP_201330	Rab GTPase A4a	24.8	5.7	37	437	10	3.41
25r	AAM62772	*Myo*-inositol monophosphatase	29.2	5.42	20	260	7	2.69
Protein translation and proteolysis								
34r	NP_187531	60S acidic ribosomal protein RPP0B	34.1	5	14	289	7	2.42
27r	AAC32062	20S proteasome subunit PAF1	30.3	4.97	19	218	6	3.28
22r	NP_188902	Proteasome subunit PRGB	22.5	5.95	16	139	4	2.62
Ion homeostasis								
16r	NP_56777	Band 7 protein	45	6.35	28	481	12	3.96
21r	NP_173451	Vacuolar ATP synthase B3	36.3	4.84	8	228	4	appear
Cell wall biosynthesis								
18r	NP_186872	RGP1	40.6	5.61	15	318	7	appear
Unknown								
14r	NP_188925	Unknown protein	27.1	5.84	16	259	5	2.69
26r	NP_001031546	Unknown protein	29.4	5.04	23	322	7	2.23

to superoxide radicals. These are, to some degree, detoxified by discrimination into oxygen and hydrogen peroxide (superoxide dismutase). The latter product is also toxic, but serves as an important signal for gene expression, leading either to programmed cell death or to the production of radical scavenging proteins. Balancing of production and detoxification of ROS is thus important for maintaining cellular functions (Apel and Hirt, 2004). For scavenging, plants possess a large number of antioxidant enzymes (Mittler *et al.*, 2004). Upon exposure to environmental stimuli and oxidative stress, it has been shown that transcripts of enzymes involved in ROS scavenging are induced. However, for the purpose of signalling, where small fluctuations of ROS are enough to activate transduction pathways, ROS production and scavenging enzymes are likely to be regulated by post-translational modifications. The following are found to be altered in the degree of phosphorylation within 30 min of exposure: thioredoxin reductase, glutathione peroxidase, ascorbate peroxidase, ATP sulphurylase, alcohol dehydrogenase, P23 co-chaperone, and HSP83 (all random positioning), protein disulphide isomerase/aldehyde dehydrogenase, GrpE like protein, and the chaperones CNP60 A and B (all 8 *g*).

Glutathione peroxidase, ascorbate peroxidase, and thioredoxin reductase are essential in the response to abiotic stresses (Milla *et al.*, 2003; Serrato *et al.*, 2004; Davletova *et al.*, 2005*a*). Glutathione peroxidase is known to be activated in animal systems by phosphorylation of tyrosin residues as a cellular response to oxidative stress (Cao *et al.*, 2003). Ascorbate peroxidase is a target protein of 14-3-3 like proteins (newly phosphorylated upon 30 min of RP) (Roberts *et al.*, 2002).

Table 2. Identification of proteins from *Arabidopsis thaliana* callus cells that are differentially phosphorylated (>2-fold up or down, $P < 0.05$) after 30 min of exposure to 8 *g*

For abbreviations see Table 1.

Spot no.	NCBI no.	*Arabidopsis* protein name	MW (kDa)	pI	Sequence coverage	Mascot score	No matched peptides	Ratio 8 *g*/control
ROS related								
2	NP_190383	Aldehyde dehydrogenase	56.4	7.11	17	661	12	0.38
16	CAB36524	GrpE like protein	34.2	5.03	23	269	7	0.41
13	NP_175945	CPN60B	63.8	6.21	10	386	8	0.35
12	NP_180367	CPN60A	62	5.09	13	519	9	0.4
Metabolism								
11	AAB60731	Phosphoglycerate mutase	62.6	5.36	24	895	17	0.44
9	NP_181192	Enolase	47.7	5.54	11	231	5	0.5
10	AAF79891	Alanine aminotransferase	51.7	5.39	17	396	9	0.47
General signalling								
14	NP_172194	Ran GTP binding	25.6	4.91	40	453	11	2.54
17	AAM65591	WD 40 repeat protein	46.6	4.66	11	236	5	3.19
Ion homeostasis								
3	NP_567778	Band 7	45	6.35	31	817	15	0.45
Protein translation and proteolysis								
8	AAM65265	60S acidic ribosomal protein POC	34.4	5.15	11	180	4	2.23
6	NP_850500	Metalloendopeptidase	59.6	6.45	20	706	14	2.13

The heat shock proteins/chaperones found to be differentially phosphorylated [protein disulphide isomerase, p23 co-chaperone and heat shock protein 90 (fragment)] are involved in protein folding, assembly, translocation, and refolding under stress conditions (reviewed by Wang *et al.*, 2004). HSP 90 takes also part in signalling cascades (Louvion *et al.*, 1998). Protein disulphide isomerase also belongs partly to this group. It is a main phosphoprotein of the endoplasmatic reticulum (Quemeneur *et al.*, 1994) and possesses two interrelated activities: as an oxidoreductase, it can catalyse the formation, reduction, and isomerization of disulphide bonds; as a polypeptide binding protein, it can function as a molecular chaperone which assists the folding of polypeptides. Transient association of PDI with nascent polypeptides during their folding prevents non-productive interactions, and thus increases the yield of correctly folded protein molecules.

Interestingly, in a recent proteomic study, it was found that some of these proteins increased in total amount starting from 2 h of exposure to hypergravity (Barjaktarovic *et al.*, 2007). Thus, modulation is a first and fast response, followed by an increase in amount of these ROS related stress proteins.

Signal transduction

Five proteins, which are well known to function in signal transduction pathways, were identified in this study. 14-3-3 like protein, the Rab GTPase homologue A4a, and myo-inositol monophosphatase were differentially phosphorylated after 30 min of exposure to random positioning. The Ran-binding protein 1a and the putative WD-40 repeat protein responded to 30 min of 8 *g*.

14-3-3 like proteins (originally isolated from mammalian brain and named by Moore and Perez in 1967) play important roles in many pathways that are regulated by phosphorylation. This is achieved by 14-3-3 protein-specific recognition of the phosphorylated target protein which completes its functional structure. 14-3-3 proteins have regulatory functions in many cellular processes such as signalling, transcription, cell division, metabolism, and vesicular transport (Roberts, 2000; Comparot *et al.*, 2003). Overexpression of 14-3-3 proteins improves stress tolerance in *Arabidopsis* (Yan *et al.*, 2004). There are 13 genes in *Arabidopsis* that code for 14-3-3 proteins. In the present study, a GF14 nu (general regulatory factor 7) family member in a highly phosphorylated state was identified where the corresponding non-phosphorylated protein spot was missing. Several protein kinases are able to phosphorylate 14-3-3 proteins in mammals and plants (Dubois *et al.*, 1997). However, phosphorylation of 14-3-3 inhibits their interaction with target proteins (Roberts, 2000) which in our case would implicate a down-regulation of interaction. We do, however, not know what the target proteins of this 14-3-3 protein are, and how many of the respective genes are expressed in cell cultures. Thus, it is not yet possible to evaluate the impact of this phosphorylation on signalling.

Inositol 1,4,5 triphosphate also plays a critical role in gravity signalling (Perera *et al.*, 2006). *Myo*-inositol monophosphatase catalyses the dephosphorylation of *myo*-inositol to form inositol, which is a precursor of two important phosphoinositol (PI) signalling messengers, diacylglycerol and myo-inositol-1,4,5-triphosphate (IP_3). Diacylglycerol activates protein kinase C which, in turn, regulates many proteins by phosphorylation. In addition, IP_3 binds to Ca^{2+} channels to release calcium from intracellular stores. Unfortunately, little

is known about the regulation of *myo*-inositol monophosphatase. A study by Berggård *et al.* (2002) revealed its activation by calbindin, a calmodulin-like protein.

Rab proteins are GTP-binding proteins belonging to the Ras superfamily and are involved in membrane trafficking pathways. Rab A4b was shown to play an important role in the polarized growth of pollen tubes and root hair tips (Preuss *et al.*, 2004; Cole and Fowler, 2006), and cell walls have been shown to be modified by altered gravitation (see Section, 'Cell wall biosynthesis'). Rab family members are phosphorylated on Ser, Thr, and Tyr residues (Plana *et al.*, 1991; Overmeyer and Maltese, 2005). Phosphorylation of the tyrosine residue in Rab24 leads to a reduced GTPase activity. Ran is another GTPase from the Ras superfamily which is involved in trafficking of RNA and proteins across nuclear pores, and in cell division (Yang, 2002). The gravitational signal should thus interact with such transport processes.

WD40 repeats (β-transducin repeats) are proteins with conserved domains of 40 amino acid motifs, terminating with Trp-Asp (W-D) and Gly-His dipeptides with 4–16 repeating units. A common function of WD40 repeats is the facilitation of protein–protein interactions, thereby forming regulatory complexes with, for example, transcription factors (Ramsay and Glover, 2005). It was shown recently that a gravitation-related expression of transcription factors can also occur within minutes (Martzivanou *et al.*, 2006; Babbick *et al.*, 2007).

Metabolism

It has been known for a long time that the alteration of gravitational forces results in metabolic responses in plant cells. (Obeland and Brown, 1994; Vasilenko and Popova, 1996; Hampp *et al.*, 1997; Wang *et al.*, 2006). These, for example, include alterations in the energy and redox state of the cells, as well as in carbohydrate metabolism. Therefore, it is not surprising that many of the enzymes which are differentially stained with Pro-Q Diamond after 30 min of exposure to random positioning are involved in primary metabolism (glycolysis: fructose-1,6-*bis*phosphate aldolase, triose phosphate isomerase, phosphoglycerate mutase). In addition, 6-phosphogluconolactonase and alanine aminotransferase (alanin catabolism) were found differentially phosphorylated. Fructose-1,6-bisphosphate aldolase and alanine aminotransferase were dephosphorylated compared to stationary control, while triose phosphate isomerase, 6-phosphogluconolactonase, and phosphoglycerate mutase were phosphorylated.

6-Phosphogluconolactonase is the second enzyme of oxidative pentose phosphate pathway (OPPP). The OPPP provides important intermediates for nucleotide biosynthesis, and NADPH for enzymes regulating the redox state of the cell (glutathione/glutaredoxin and thioredoxin systems) (Grant, 2008).

The responses to hypergravity were different. First, fewer glycolytic enzyme proteins altered in phosphorylation were found. Second, the amount of phosphorylated alanine aminotransferase was decreased as shown for RP, while phosphoglycerate mutase, in contrast to RP, was also less phosphorylated. Third, enolase showed decreased phosphorylation. Enolase is located at the converging point between glycolysis and gluconeogenesis and catalyses the conversion of 2-phosphoglycerate to phospho*enol*pyruvate. It is activated by dephosphorylation (Lal *et al.*, 1998; Forsthoefel *et al.*, 1995) which infers an up-regulation of this step in glycolysis. In our previous studies evidence was also found for increased rates of glycolysis and anaplerosis under altered *g* conditions (Hampp *et al.*, 2001; Martzivanou and Hampp, 2003).

Protein translation and proteolysis

Two ribosomal proteins have been identified, 60S acidic ribosomal protein P0-B (random positioning) and 60S acidic ribosomal protein P0-C (8 *g*) with increased phosphorylation after treatment. Ribosomal P-proteins are acidic proteins, that interact with tRNA, mRNA, and elongation factors during translation. Phosphorylation of these proteins leads to increased (selective) translation activity (Bailey-Serres *et al.*, 1997; Aguilar *et al.*, 1998). This is in accordance with increased gene expression which can start within minutes after altering the gravitational field (e.g. transcription factors, kinases etc; Moseyko *et al.*, 2002; Kimbrough *et al.*, 2004; Martzivanou *et al.*, 2006), and with increased amounts of ribosomal proteins upon exposure to clinorotation (Wang *et al.*, 2006). In addition, two subunits of proteasome 20S (PBD1 and PAF1) exhibited increased phosphorylation in response to random positioning, and a metalloendopeptidase to 8 *g*. Proteasomes are important for protein turnover, and their phosphorylation regulates activity and structure (Umeda *et al.*, 1997; Bose *et al.*, 2004). Metalloendopeptidases are, in most cases, Zn-binding proteases which catalyse the hydrolysis of non-terminal peptide bonds, especially those with hydrophobic residues.

Cell wall biosynthesis

Reversibly glycosylated polypeptides (RGPs) are highly conserved plant proteins, probably involved in plant polysaccharide synthesis (Delgado *et al.*, 1998). Self-glycosylation of RGPs increases their ability to become a part of protein complexes (De Pino *et al.*, 2007). On the other hand, phosphorylation reduces self-glycosylation by decreasing their affinity to sugars. RGP1 responded to random positioning by an increase in the phosphorylation status. RGPs are also differentially expressed after long-term exposure to hypergravity (Z Barjaktarović, data not shown), or in response to clinorotation (Wang *et al.*, 2006). There is also a lot of other data, showing that the expression of cell wall-related proteins is well affected by altered gravitational fields (Martzivanou and Hampp, 2003; Hoson *et al.*, 2003). Thus, if RGPs are also involved in cell wall biosynthesis, these data would give further support to the notion that altered *g* affects cell wall biosynthesis.

Ion homeostasis

Protons are implicated in plant signal transduction as second messengers (Roos *et al.*, 1998). One of the early

responses to gravistimulation is alkalinization of the cytosol (Fasano *et al.*, 2001; Johannes *et al.*, 2001). pH oscillations could be a result of the activation of plasma membrane or vacuolar H^+-ATPases (Perrin *et al.*, 2005). Vacuolar (V) ATPase appears as a phosphorylated protein upon exposure to random positioning. The spot was not detectable in control samples. In animal systems, the C subunit of V-ATPase is activated by phosphorylation through protein kinase A (Rein *et al.*, 2008). In barley, subunits A and B of V-ATPase are targets of 14-3-3-proteins (see above). 14-3-3 binding leads to the activation of the V-ATPase (Klychnikov *et al.*, 2007). This finding could thus indicate that V-ATPase could play a role in proton homeostasis and early signalling as a response to changes in the gravitational force.

Band 7 protein, which probably also functions in ion homeostasis, responded after both treatments (RP increased phosphorylation, 8 *g* decreased phosphorylation; distinct spots). In a study by Davletova *et al.* (2005*b*), the transcript level of band 7 protein was up-regulated upon oxidative- and light stress. In our proteome study, the expression of this protein was also increased upon long-term exposure to 8 *g* (data not shown).

Conclusions

Steps of possible signalling cascades

Proteins found to be altered in the degree of phosphorylation can be grouped as follows. One group (thioredoxin reductase, ascorbate peroxidase, glutathione reductase, glutathione-*S*-transferase, aldehyde dehydrogenase, FQR1, lipoamide dehydrogenase) is involved in the detoxification of reactive oxygen species (ROS) (Fig. 3). We thus conclude that changes in the gravitational field (i.e. increased gravitation, as well as compensation of gravitation) cause the formation of ROS, the production of which is balanced by the activation/increased expression of these enzymes.

ROS such as H_2O_2 can activate kinases of signalling cascades (de la Fuente van Bentem *et al.*, 2008). In accordance, the second group of proteins represents members of signalling chains (Fig. 3). MAPkinases, for example, interact with transcription factors such as WRKYs etc. which have also been shown to respond to changes in the gravitational field (Martzivanou *et al.*, 2006; Babbick *et al.*, 2007). Furthermore, many of the proteins altered in expression are Ca^{2+} dependent (Barjaktarovic *et al.*, 2007). There is evidence (not shown) that changes in cytosolic Ca^{2+} upon

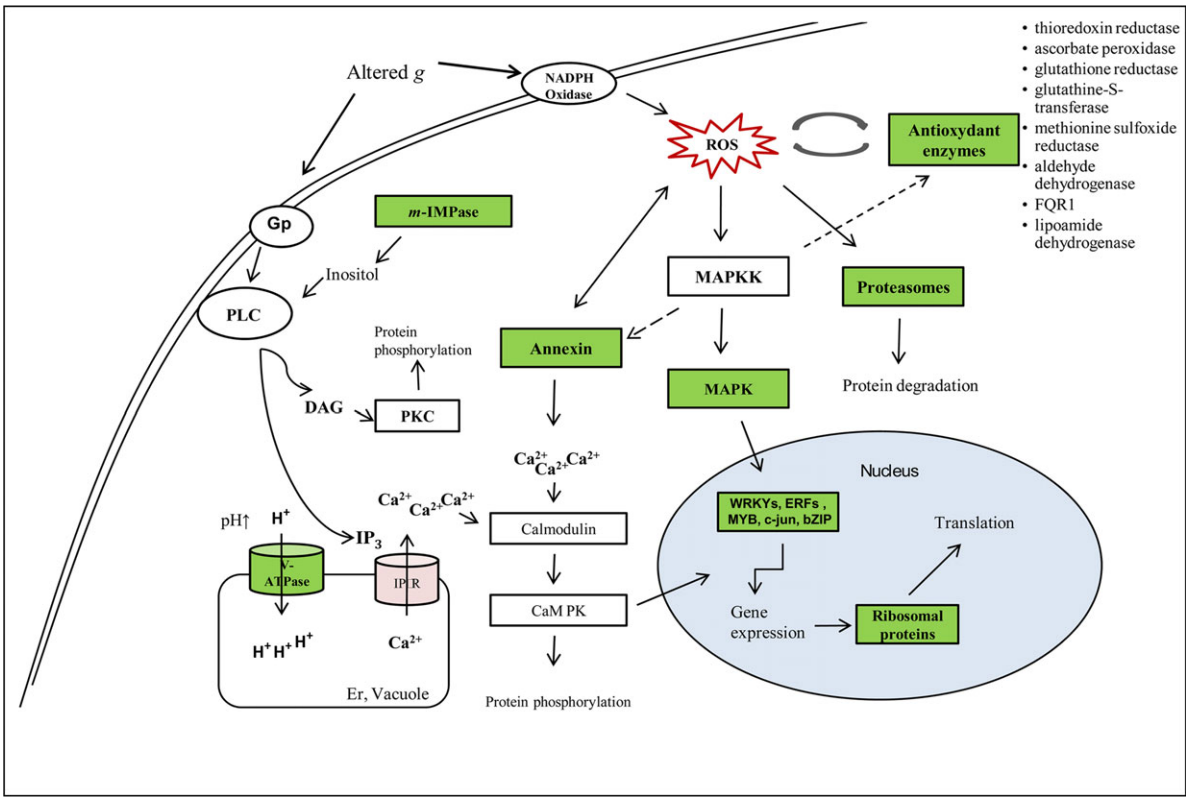

Fig. 3. Summary of the pathways described in this study. Green boxes represent some of the proteins identified in this work. Transcription factors WRKYs, ERFs, bZIP were analysed by Babbick *et al.* (2006, 2007). MAPK, mitogen activated protein kinases; ROS, reactive oxygen species; CaMPK, calmodulin-dependent protein kinase; *m*-IMPase, *myo*-inositol monophosphatase; V-ATPase, vacuolar ATPase; IP_3-R, inositol 1,4,5 triphosphate-gated calcium channels; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; Gp, G proteins.

gravitational stimulation result from internal Ca^{2+} sources; thus, membrane signals via phosphatidyl inositol (PLC, DAG, IP_3) could be involved by opening Ca^{2+} channels (IP_3R). Altogether, this results in enhanced activities in primary metabolism in order to adjust to the altered environment.

Hypergravity versus random positioning

In addition to clinorotation and magnetic levitation, random positioning is increasingly used as a means of simulating microgravity responses. A comparison of gene expression between callus cells exposed to random positioning and to sounding rocket microgravity revealed some similarities (Babbick *et al.*, 2007). This kind of exposure was used as an approach to identify responses towards a reduction in the effective gravitational field. Hypergravity, on the other hand, induces clear responses compared with 1 *g* controls which are well documented in the literature. By comparing data from both approaches we were searching for treatment-specific responses.

Interestingly, the number of proteins altered in the degree of phosphorylation was higher after exposure to random positioning compared to hypergravity. In a recent study, where the respective effects on the total proteome were investigated, the opposite was found. Here, the number of specific proteins with altered amounts was clearly higher after exposure to hypergravity than to random positioning (Barjaktarovic *et al.*, 2007). We thus speculate, that random positioning causes a 'mild type' of stress, which can be met by protein modulation, while hypergravity acts more severely, inducing protein expression. As some homologies in gene and protein expression were found between sounding rocket microgravity and random positioning/magnetic levitation (Babbick *et al.*, 2007; Barjaktarovic *et al.*, 2007), the responses to random positioning could also apply for real microgravity. This has to be tested in orbit.

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