Manuscript Details

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Title	OVEREXPRESSION OF 14-3-3 PROTEINS ENHANCES COLD TOLERANCE AND INCREASES LEVELS OF STRESS-RESPONSIVE PROTEINS OF ARABIDOPSIS PLANTS
Article type	Research Paper

Abstract

14-3-3 proteins are a family of conserved proteins present in eukaryotes as several isoforms, playing a regulatory role in many cellular and physiological processes. In plants, 14-3-3 proteins have been reported to be involved in the response to stress conditions, such as drought, salt and cold. In the present study, 14-3-3ε and 14-3-3ω isoforms, which were representative of ε and non-ε phylogenetic groups, were overexpressed in Arabidopsis thaliana plants; the effect of their overexpression was investigated on H+-ATPase activation and plant response to cold stress. Results demonstrated that H+-ATPase activity was increased in 14-3-3ω-overexpressing plants, whereas overexpression of both 14-3-3 isoforms brought about cold stress tolerance, which was evaluated through ion leakage, lipid peroxidation, osmolyte synthesis, and ROS production assays. A dedicated tandem mass tag (TMT)-based proteomic analysis demonstrated that different proteins involved in the plant response to cold or oxidative stress were over-represented in 14-3-3ε-overexpressing plants.

Keywords	14-3-3 proteins; plant proteomics; H+-ATPase; cold stres.

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FIGURE 1.tif [Figure]

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TOR VERGATA
UNIVERSITÀ DEGLI STUDI DI ROMA

Macroarea di Scienze Matematiche, Fisiche e Naturali
Dipartimento di Biologia

Rome, July 22th 2019

Dear Editor,

please find enclosed a revised version of the manuscript entitled "Overexpression of 14-3-3 proteins enhances cold tolerance and increases levels of stress-responsive proteins of Arabidopsis plants", manuscript number PSL_2019_481.

Yours Sincerely,

Sabina Visconti Chiara D'Ambrosio

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Dear Editor,

please find enclosed a revised version of the manuscript entitled "Overexpression of 14-3-3 proteins enhances cold tolerance and increases levels of stress-responsive proteins of Arabidopsis plants", manuscript number PSL_2019_481.
We would like to thank the Reviewers for the positive feedback and the constructive criticisms. In this revised version, most of Reviewers' suggestions and critical points have been addressed. In particular:

Reviewer 1
The literature review on the subject in the current submission is not updated as more 14-3-3s studied in additional plant species including cereals such as wheat and Brachypodium are not reported.
Answer. As suggested, novel references about wheat and Brachypodium have been added.

Authors claim that 14-3-3ω- and 14-3-3ε-overexpressing lines displayed a 3 to 1 ratio, suggesting the presence of a single T-DNA insertion. It is better to include a Southern blot analysis not only to confirm that the insert is present in these lines as a single copy but also to check the integrity of the transgene encoding a fusion protein 14-3-3ω:YFP or 14-3-3ε:YFP.
Answer. Reviewer 1 suggested to include a Southern blot analysis to evaluate T-DNA insertions. We think that what was relevant for the purpose of this paper was to ascertain that levels of overexpressed *14-3-3ω* and *14-3-3ε* were comparable. This issue was demonstrated by western blotting experiments. To avoid any misinterpretation, the sentence about the segregation analysis has been removed from the text.

The quality of the western blot is not convincing with too bright and low resolution image.
Answer. Quality of western blotting has been improved, as shown in the novel version of Fig. 1.

There is no data showing the phenotypic comparison between WT and 14-3-3 overexpressing lines under cold stress while authors report that these lines are more tolerant to this type of stress.
Answer. As specifically stated in the novel version of the manuscript, no major phenotypical differences were detected after 18 h of cold treatment. We think that this fact is conceivable, considering that the time used for treatment (18 h) is a too short to induce visible damages on plants. However, injuries at the cellular level were clearly induced, as shown by ion leakage and lipid peroxidation analyses.

According to immunoblot and Subcellular localization data, 14-3-3ω isoform is associated to plasma membrane. Authors should indicate whether there is a marked sequence difference between the two 14-3-3, explaining the detection of one isoform in the PM but not the other.
Answer. The text has been modified in order to clarify this point. 14-3-3ω localized at the plasma membrane as the consequence of its association with the plasma membrane H^+-ATPase. Conversely, 14-3-3ε is not able to bind the H^+-ATPase and therefore cannot localize at the plasma membrane. The different abilities of the two 14-3-3 isoforms to interact with H^+-ATPase have already been demonstrated in previous papers (Palluca et al, 2014).

Proteomic analysis was performed to identify cold stress associated proteins. This study was carried out on a 14-3-3ε and not 14-3-3ω overexpressing line. The rationale for this choice (explained in page 14/lanes 311-314) was based on the fact that the over- expression of the 14-3-3ω stimulates the plasma membrane H+-ATPase, that could per se alter gene expression and hence may have an overlapping effect on cold stress. This rationale is not clear. In my opinion, it would be even more informative to make analysis on both types of transgenic lines and see whether there are any specific proteins required for cold stress response and linked to the plasma membrane H+-ATPase.

Answer. We agree with Reviewer 1 that it would have been interesting to extend the proteomic analysis on both 14-3-3ω and 14-3-3ε over-expressing lines. Unfortunately, available funds were not sufficient to perform TMT-proteomic analysis of wild type and the two transgenic lines, under control and stress conditions. This condition is still persisting. As already reported in the text, the rationale for the choice of 14-3-3ε is that this protein is not able to stimulate the H^+-ATPase activity. Accordingly, we avoid to perform a proteomic analysis on the effect of cold stress in a condition (14-3-3ω overexpression) of stimulation of the H^+-ATPase activation, where the observed protein concentration changes can derive by H^+-ATPase *per sé*.

It has been reported that 14-3-3l together with CRPK1 are key regulators of cold response in Arabidopsis and the overexpression of 14-3-3l lead to a reduced freezing tolerance (Liu et al., 2017). This finding was not sufficiently and critically addressed in the context of this work where ω and ε were show to exert opposite regulatory role in cold tolerance compared to l isoform

Answer. The point raised by Reviewer 1 has been better discussed and critically addressed in the new version of the manuscript.

Authors should discuss why antioxidant enzymes such as SOD, POX and CAT were not detected in this proteomic study although both 14-3-3ω-and 14-3-3ε-overexpressing lines exhibit less oxidative damage (as it was assessed by measuring H202 and MDA) under cold stress?

Answer. As suggested by the Reviewer 1, these two points have been critically discussed in the novel version of the manuscript.

Authors should also discuss how the 14-3-3 overexpression leads to an increase in proline contents.

Answer. This information has been added in this revised version.

Reviewer 2

A comparison, using the same proteomic approach, between the plants overexpressing ε and those overexpressing ω could have been very informative.

Answer. We agree with Reviewer 1 that it would have been interesting to extend the proteomic analysis on both 14-3-3ω- and 14-3-3ε-overexpressing lines. Unfortunately, available funds were not sufficient to perform TMT-proteomic analysis of wild type and the two transgenic lines, under control and stress conditions. This condition is still persisting. As already reported in the text, the rationale for the choice of 14-3-3ε is that this protein is not able to stimulate the H^+-ATPase activity. Accordingly, we avoid to perform a proteomic analysis on the effect of cold stress in a condition (14-3-3ω overexpression) of stimulation of the H^+-ATPase activation, where the observed protein concentration changes can derive by H^+-ATPase *per sé*.

The authors should also mention if, among the differently represented proteins, they have found putative or known 14-3-3-binding proteins.
Answer. As suggested, we verified the occurrence of putative 14-3-3 binding sites in the differentially represented proteins. As reported in the novel version of the manuscript, only one protein, namely Dormancy-associated protein 1, contains a canonical 14-3-3-binding site. This protein was down-represented to the same extent in wild type and 14-3-3ε-overexpressing plants upon cold treatment.

It is not clear if the proteomic data presented here are from only one analysis of control/treated plants, or if they come from at least two independent biological replicates. This point should be clarified in the manuscript and the reproducibility of the obtained results should be mentioned.
Answer. Proteomic data come from two independent biological replicates of wild type and 14-3-3ε-overexpressing plants. This has been clearly reported in this amended version of the manuscript, which also contains the error associated with each protein measurement (see Supplementary Table S1).

This study has been performed with two lines overexpressing two distinct 14-3-3s: phenotypes (or absence of visible phenotype) of these lines in control/cold conditions are not mentioned or described in this manuscript.
Answer. No phenotypical differences were observed between wild type and 14-3-3-overexpressing plants both under control and cold stress conditions. This has now been clearly reported in this amended version of the manuscript.

Figure 1A and B: the blot images are a little bit blurry, and molecular weight markers are missing (also in Figure 2B). In Figure 1A, there is a signal in the middle (low part) of the immunoblot (between lanes 14-3-3ω L3 and WT) that seems to come from a cut band: if the image in Figure 1A is from different blots, this should be shown/indicated.
In Figure 1 (A and B) and in Figure 2B, there is no control of equal loading. This should be added, especially in Figure 2B because the authors claim that the 14-3-3ω isoform is more associated to membrane fraction than the 14-3-3ε one (p.12, line 265): western blotting performed on the same samples with an antibody directed against a plasma membrane protein (such as the H^+-ATPase) is needed to support this statement by showing equal loading of the different plasma membrane preparations. Moreover, western blotting with anti-14-3-3 antibodies would also have been informative to additionally reveal the endogenous 14-3-3s associated with the plasma membrane fractions purified from 14-3-3ω- and 14-3-3ε-overexpressing lines.
Answer. Figure 1 has been modified according to the Reviewer's suggestion. Molecular weight markers and equal control loading have been added, as requested.
The image in Fig. 1A comes from one blotting in which a central band (recombinant YFP protein used as positive control for Ab binding) was removed. We here below are including the original image.

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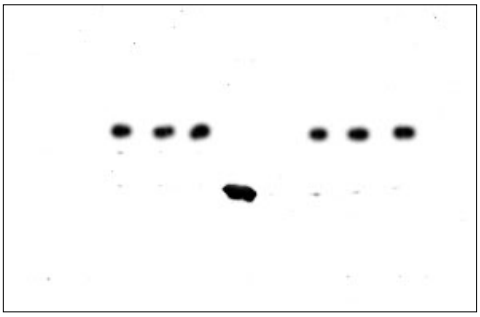

Figure 3: the data presented in this Figure are not sufficient to claim that both isoforms are localized in the cytosol and that ω is also present at the plasma membrane (p13, line 275): data showing co-localization of the proteins with markers of these compartments should be added. Moreover, several previous studies have described the subcellular localization of 14-3-3ω and 14-3-3ε (both also detected in the nucleus): the authors should mention and discuss this point in the manuscript (section 3.3), and cite previous studies. Scale bars should also be added in Figure 3.
Answer. As suggested, a new experiment showing colocalization of 14-3-3ω with the marker of the plasma membrane has been performed, and included as Figure 3 in the novel version of the manuscript. Moreover, the subcellular localization of 14-3-3 isoforms has been discussed and previous articles on this issue have been cited. Scale bar has been added.

Figure 4A: how do the authors explain that values of conductance of both 14-3-3ω- and 14-3-3ε-overexpressing plants are significantly lower than that of WT plants in control condition?
Answer. Reasons for the differences in the REC values between wild type and 14-3-3-overxepressing plants under control conditions are actually unclear to us.

Figure 6: the image showing the STRING analysis is blurry and GRF10 (ε) is difficult to spot in this figure.
Answer. STRING images are often blurry due to a number of objects present in it. This condition is worst when not a real image is observed but its conversion into the pdf, however we tried to improve this image. An improvement of GRF10 spotting was also obtained by highlighting the corresponding knot with an arrow.

Table 1: the authors should define the sum PEP score and PSMs in the legend
Answer. PEP and PMSs have been defined in the legend, as requested.

Minor points to be addressed
Answer. Minor points at pages 1, 12, 23 and 24 have been addressed, as requested.

Yours Sincerely,

Sabina Visconti Chiara D'Ambrosio

Highlights

- 14-3-3ε and ω isoforms differentially regulate the H^+-ATPase
- Overexpression of 14-3-3ε and ω increases cold tolerance in *Arabidopsis* plants
- TMT-proteomics reveals stress-related proteins regulated by 14-3-3ε-overexpression

OVEREXPRESSION OF 14-3-3 PROTEINS ENHANCES COLD TOLERANCE AND INCREASES LEVELS OF STRESS-RESPONSIVE PROTEINS OF ARABIDOPSIS PLANTS

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Keywords: 14-3-3 proteins; plant proteomics; H^+-ATPase; cold stress

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Abstract

14-3-3 proteins are a family of conserved proteins present in eukaryotes as several isoforms, playing a regulatory role in many cellular and physiological processes. In plants, 14-3-3 proteins have been reported to be involved in the response to stress conditions, such as drought, salt and cold. In the present study, 14-3-3ε and 14-3-3ω isoforms, which were representative of ε and non-ε phylogenetic groups, were overexpressed in *Arabidopsis thaliana* plants; the effect of their overexpression was investigated on H^+-ATPase activation and plant response to cold stress. Results demonstrated that H^+-ATPase activity was increased in 14-3-3ω-overexpressing plants, whereas overexpression of both 14-3-3 isoforms brought about cold stress tolerance, which was evaluated through ion leakage, lipid peroxidation, osmolyte synthesis, and ROS production assays. A dedicated tandem mass tag (TMT)-based proteomic analysis demonstrated that different proteins involved in the plant response to cold or oxidative stress were over-represented in 14-3-3ε-overexpressing plants.

1. Introduction

14-3-3 proteins are a family of conserved proteins in eukaryotes that play a regulatory role in many cellular and physiological processes by direct interaction with target proteins [1]. They bind specific phosphoserine/phosphothreonine containing motifs [2-4] in the clients, thus affecting their activity, subcellular localization or stability [5,6]. In plants, 14-3-3 proteins are involved in the regulation of ion membrane transport, carbon and nitrogen metabolism, gene expression, stomatal movement, hormone signaling and in the coordination of different signal transduction pathways [7-11]. They have also been reported to be involved in the plant response to stress conditions [12-14]. In fact, environmental and biotic stimuli affect the expression levels of 14-3-3 proteins. Furthermore, many proteins involved in the response to different stresses have been shown to be 14-3-3 clients [14-16]. In particular, 14-3-3 proteins play a role in the plant response to water stress, as demonstrated by the improvement of tolerance under moderate drought stress conditions displayed by cotton plants overexpressing the *Arabidopsis* 14-3-3λ [17]. Similarly, the maize ZmGF14-6 isoform constitutively expressed in rice, enhanced tolerance to drought by strongly inducing drought-responsive rice genes [18]. Recently, 14-3-3 proteins have been reported to be involved in the salt overly sensitive (SOS) pathway, which is known to control sodium homeostasis during salt stress [19]. Accordingly, the expression of 14-3-3 isoforms BdGF14d and TaGF14b from *Brachypodium dystachion* and wheat, respectively, was shown to enhance salt tolerance in transgenic tobacco plants [20,21].

It has also been demonstrated that 14-3-3 proteins take part to the response to cold stress; in fact, Jarillo and colleagues identified in *Arabidopsis* two 14-3-3 proteins, namely RARE COLD INDUCIBLE 1A and 1B, which are induced by cold stress [22]. More recently, the *Arabidopsis* 14-3-3ψ isoform, i.e. RCI1A, has been demonstrated to function as a negative regulator of cold acclimation and freezing tolerance, by negatively controlling the expression of cold-responsive genes [23]. Similarly,

Liu and coworkers demonstrated the involvement of the *Arabidopsis* 14-3-3λ isoform in the negative regulation of the stability of C-repeat binding factor (CBF) proteins that play a pivotal role in freezing tolerance [24].

14-3-3 proteins exist as multiple isoforms; eight isoforms are expressed in rice [25], twelve in tomato [26], and thirteen in *Arabidopsis* [27], where they are designed by Greek letters and divided into two major groups (named ε and non-ε), based on phylogenetic analysis. The high degree of amino acid sequence identity and the conserved three-dimensional structure shared by 14-3-3 isoforms [28-31] suggest a functional redundancy. On the other hand, growing evidence demonstrates that 14-3-3 isoforms display different affinities in the interaction with specific targets [32,33], even though residues directly involved in binding are absolutely conserved in all isoforms [34]. A proteomic study aimed at identifying 14-3-3 binding clients in developing *Arabidopsis* seeds demonstrated that 14-3-3 target proteins differentially interact with 14-3-3ε and χ isoforms [35]. More recently, Pallucca et al. [36] demonstrated the existence of an isoform specificity in the 14-3-3 binding to a phosphopeptide array reproducing different 14-3-3 binding sites of protein clients. In the same study, it was also demonstrated that *Arabidopsis* 14-3-3 isoforms bind the H^+-ATPase with different affinities *in vitro*. Non-ε 14-3-3 isoforms were more active in the interaction and activation of the enzyme, when compared to 14-3-3 isoforms belonging to the ε group.

In the present study, we generated *Arabidopsis* plants overexpressing 14-3-3ε and 14-3-3ω isoforms, as representative of ε and non-ε group, respectively. Then, the effect of 14-3-3ε and 14-3-3ω overexpression on the regulation of the H^+-ATPase activity and on the plant response to cold stress conditions was investigated. Moreover, the consequence of cold stress on the protein repertoire of 14-3-3-overexpressing plants was studied by a quantitative proteomic analysis.

2. Materials and methods

2.1 Plant growth and low temperature treatment

Wild-type (WT) and transgenic *Arabidopsis thaliana* plants [ecotype Columbia (Col-0)] were grown in soil or in Murashige and Skoog (Duchefa, the Netherlands) medium in a growth chamber at 23 °C, 80% humidity, under a 16 h light/8 h dark cycle. Five-week old plants were subjected to cold treatment by incubating them in the dark, at 4 °C, for 18 h. Control plants were incubated at 23 °C under the same conditions, in the dark.

2.2 Vector construction and Agrobacterium-mediated transformation of Arabidopsis plants

The 14-3-3ε and 14-3-3ω coding sequences were cloned upstream of the YFP in a modified pGreen 0029 binary vector containing a double 35S promoter and the translational enhancer sequence of TEV [37]. Both 14-3-3 coding sequences were PCR-amplified to be inserted in the *Nco*I restriction site located at the 5' end of YFP sequence using the following primers: 14-3-3ε fw primer 5'-CCATGGAGAATGAGAGGGAAAAGC-3' and 14-3-3ε rev primer 5'-CCATGGGGTTCTCATCTTGAGGC-3'; 14-3-3ω fw 5'-CCATGGCGTCTGGGCGTGAA-3' and 14-3-3ω rev primer 5'-CCATGGGCTGCTGTTCCTCGG-3' (where *Nco*I restriction site is underlined) and the cDNAs previously obtained [36] as the templates. PCR-amplified cDNAs were controlled by DNA sequencing (Eurofins Genomics, Ebersberg, Germany) before proceeding to *Agrobacterium* transformation. Modified pGreen0029 binary vectors were introduced through electroporation in the GV3101 *Agrobacterium* strain, which harbors the pSOUP vector, by using a Micropulser Electroporation apparatus (Bio-Rad, Hercules, CA).

A. thaliana ecotype Col-0 plants were transformed by floral dip method [38]. Transgenic seedlings were selected on MS medium containing 50 µg/ml kanamycin and then grown in soil. T2 lines were selected for antibiotic resistance and the presence of transgenic construct was confirmed by PCR

analysis using primers specific for 35S promoter (35S fw primer 5'-GTCTCAGAAGACCAAAGGGC-3'; 35S rev primer 5'-CCTCTCCAAATGAAATGAACTTCC-3'). The expression of transgenic constructs was verified by YFP fluorescence.

2.3 Protoplast transformation and confocal microscopy

Arabidopsis protoplasts were isolated and transformed as described by Yoo et al. [39]. Briefly, 20 *Arabidopsis* leaves were cut into 0.5-1 mm strips and submerged in 10 ml of a solution containing 1 % cellulase R10, 0.25% macerozyme R10, 0.4 M mannitol, 20 mM KCl, 10 mM $CaCl_2$, 20 mM MES, pH 5.7. After 30 min of vacuum infiltration, enzymatic digestion was extended for 3 h without shaking, in the dark. Protoplasts were then recovered by filtration on a 100 µm nylon mesh, centrifuged for 2 min at 100 x g, and washed twice in W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7) before resuspending in 0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7. For transient transformation, 20 µg of plasmid DNA were added to 10^4 protoplasts in 40% PEG4000, 0.2 M mannitol, 0.1 M $CaCl_2$. After 30 min of incubation at 23 °C, samples were diluted with W5 solution, and centrifuged to remove PEG. For plasma membrane staining, 1x Cell Mask Orange (Thermo Fisher Scientific, USA) was added to protoplasts suspension. YFP fluorescence was revealed by confocal microscopy after 18 h with a laser scanning microscope (Olympus FV1000). Lasers at 488 nm (argon), 635 nm (diode) and 554 nm were used to detect YFP, chlorophyll and Cell Mask Orange signals, respectively. Images of 512 × 512 pixels were acquired using a 20× objective.

2.4 Purification of plasma membrane from Arabidopsis leaves

Two-phase partitioned plasma membranes were purified from 100 g of 5-week old *Arabidopsis* seedlings as previously described [40].

125 *2.5 Phosphohydrolytic activity*

126 The phosphohydrolytic activity was assayed according to Camoni et al. [41], using 50 µg of plasma
127 membrane proteins. Experiments were carried out using plasma membrane preparations obtained
128 by pooling 5-week old *Arabidopsis* plants of three T3 homozygous lines.

129

130 *2.6 Protein quantification*

131 Protein concentration was determined by the method of Bradford, using bovine serum albumin as
132 the standard [42].

133

134 *2.7 SDS-PAGE and Immunoblotting*

135 SDS–PAGE was performed as described by Laemmli [43], using a Mini Protean apparatus (Bio-Rad,
136 Hercules, CA). For immunoblotting analysis, proteins were separated by SDS–PAGE, then
137 electroblotted onto a PVDF membrane with 48 mM Tris, 39 mM glycine, 0.1% SDS and 10%
138 methanol. After blocking for 1 h in Tris buffered saline containing 0.05% Tween-20 and 5% no-fat
139 dried milk at room temperature, the membrane was incubated with anti-14-3-3 antibodies (1:1000)
140 [44] or anti-YFP antibodies (Santa Cruz Biotechnology, Dallas, TX) (1:200). After washing with TBS
141 containing 0.05% Tween-20, the membrane was incubated with horseradish peroxidase-conjugated
142 anti-rabbit secondary antibody 1:5000 (Bio-Rad, Hercules, CA). As loading control, PVDF membranes
143 were immunodecorated with anti-actin antibodies (Sigma-Aldrich, USA), used 1:1000.

144

145 *2.8 Electrolyte leakage and lipid peroxidation assays*

146 For the electrolyte leakage test, 0.2 g of *Arabidopsis* leaves were cut into 5 mm slices that were
147 shaken in 30 ml of deionized water, for 2 h, at 25 °C. The electrical conductivity of solution was

148 measured by using an electrical conductivity meter. Boiled samples were used to determine the
149 maximum percentage of electrolyte leakage.
150 Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) production, following the
151 protocol reported by Taulavuori et al. [45]. Briefly, 100 mg of *Arabidopsis* leaves were homogenized
152 in 2 ml of 10% trichloroacetc acid containing 0.25% thiobarbituric acid. The mixture was incubated
153 at 95 °C, for 30 min; then it was centrifuged at 10000 g for 20 min, and the absorbance of
154 supernatant measured at 532 and 600 nm.
155
156 *2.9 Determination of H_2O_2*
157 The concentration of H_2O_2 released in solution was determined with the FOX1 method [46], which
158 is based on the hydrogen peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with
159 xylenol orange dye. Thus, *Arabidopsis* leaves were cut into thin slices and incubated in deionized
160 water for 30 min. The incubation medium was then added to an equal volume of the assay reagent
161 containing 500 mM ammonium ferrous sulfate, 50 mM H_2SO_4, 200 mM xylenol orange, and 200 mM
162 sorbitol. After 45 min of incubation, the absorbance of the Fe^{3+}-xylenol orange complex was
163 measured at 560 nm, and the H_2O_2 concentration was calculated by interpolation from a standard
164 curve obtained with H_2O_2 solutions of known concentration.
165
166 *2.10 Determination of free proline*
167 Half g of *Arabidopsis* leaves was powdered in liquid N_2, and homogenized in 1 ml of 70% ethanol.
168 After centrifugation at 14000 g for 20 min, 0.5 ml of sample were added to 1 ml of a solution
169 containing 1% ninhydrin, 60% acetic acid, 20% ethanol, and incubated at 95 °C, for 20 min. The
170 absorbance of samples was measured at 520 nm, and the amount of free proline calculated by
171 interpolation from a standard curve obtained with proline solutions of known concentration.

172

173 *2.11 Statistics*

174 Statistical analysis was performed by means unpaired t student test using GraphPad Prism software.

175

176 *2.12 Protein extraction, digestion and peptide fractionation*

177 Proteins from pooled leaves of control and 14-3-3ε-overexpressing *Arabidopsis* plants of three T3
178 homozygous lines treated under normal and cold temperature conditions, i.e. WT Contr, 14-3-3
179 Contr, WT Cold and 14-3-3 Cold, respectively, were extracted in parallel through a modified version
180 of the trichloroacetic acid (TCA)-acetone precipitation method [47]. Thus, 1 g of plant leaves were
181 pulverized in parallel using a mortar containing liquid N_2, and the fine powder samples were
182 suspended in 30 ml of ice-cold acetone containing 10% w/v TCA and 0.07% w/v DL-dithiothreitol
183 (DTT), at −20 °C, overnight. Two independent biological replicates were analyzed in comparison for
184 each experimental condition assayed. The protein precipitates were pelleted by centrifugation at
185 35,000 g, for 1 h, and resuspended in 30 ml of ice-cold acetone containing 0.07% w/v DTT for 1 h,
186 at −20 °C. Protein precipitates were centrifuged again at 35,000 g for 1 h, at 4 °C, and washed three
187 times with ice-cold acetone containing 0.07% w/v DTT. Protein pellets were solubilized in 5 volumes
188 of 8 M urea, 50 mM triethylammonium bicarbonate (TEAB), pH 8.5, and added with a protease
189 inhibitors cocktail for plant tissues (Sigma-Aldrich, USA). Samples were left at 30°C for 1 h, and then
190 extracted by ultrasonication at 50 W output twice for 10 s, with a 60 s rest between bursts. Samples
191 were vortexed briefly and centrifuged at 12,000 rpm for 30 min, at 4 °C. Protein concentration in
192 samples was determined using the Pierce BCA Protein assay kit™ (Thermo Scientific, Rockford, IL,
193 USA), according to manufacturer's instructions. An aliquot of each protein sample (100 μg) was
194 adjusted to a 100 μl final volume with 100 mM TEAB, and then reduced with 5 μl of 200 mM tris(2-
195 carboxyethylphosphine), for 60 min, at 55 °C. Protein samples were then alkylated by adding 5 μl of

375 mM iodoacetamide in the dark, for 30 min, at 25 °C. Alkylated proteins were then precipitated by addition of 6 volume of cold acetone to remove chemicals. After precipitation, proteins were pelleted by centrifugation at 8,000 g, for 10 min, at 4 °C, and air-dried. Each sample was digested with freshly prepared trypsin (ratio of enzyme to protein 1:50) in 100 mM TEAB, at 37 °C, overnight. Resulting peptides from each protein sample were labelled with the TMTsixplex Label Reagent Set (Thermo-Fisher Scientific, USA) according to the matching WTContr-TMT6-126, WTCold-TMT6-127, 14-3-3Contr-TMT6-128 and 14-3-3Cold-TMT6-129, at 25 °C, in agreement to manufacturer's instructions. After 1 h of reaction, 8 µl of 5% w/v hydroxylamine was added in each tube and mixed for 15 min, in order to quench the derivatization reaction. For a set of comparative experiments, tagged peptide mixtures were mixed in equal-molar ratios (1:1:1:1) and vacuum-dried under rotation. Then, pooled TMT-labelled peptide mixtures were suspended in 0.1% trifluoroacetic acid, and fractionated by using the Pierce™ High pH Reversed-Phase Peptide fractionation kit (Thermo-Fisher Scientific) to remove unbound TMT reagents and reduce sample complexity, according to manufacturer's instructions. After fractionation, eight fractions of TMT-labelled peptides were collected, vacuum-dried and finally reconstituted in 0.1% formic acid for subsequent mass spectrometric analysis.

2.13 NanoLC-ESI-MS/MS analysis

TMT-labelled peptide fractions (eight in total number as deriving from two independent biological replicates of four different experimental conditions) were analyzed on a nanoLC-ESI-Q-Orbitrap-MS/MS platform consisting of an UltiMate 3000 HPLC RSLC nano system (Dionex, USA) coupled to a Q-ExactivePlus mass spectrometer through a Nanoflex ion source (Thermo Fisher Scientific). Peptides were loaded on an Acclaim PepMapTM RSLC C18 column (150 mm × 75 µm ID, 2 µm particles, 100 Å pore size) (Thermo-Fisher Scientific), and eluted with a gradient of solvent B

(19.92/80/0.08 v/v/v water/acetonitrile/formic acid) in solvent A (99.9/0.1 v/v water/formic acid), at a flow rate of 300 nl/min. The gradient of solvent B started at 5%, increased to 60% over 125 min, raised to 95% over 1 min, remained at 95% for 8 min, and finally returned to 5% in 1 min, with a column equilibrating step of 20 min before the subsequent chromatographic run. The mass spectrometer operated in data-dependent mode, using a full scan (*m/z* range 375-1500, nominal resolution of 70,000), followed by MS/MS scans of the 10 most abundant ions. MS/MS spectra were acquired in a scan *m/z* range 110-2000, using a normalized collision energy of 32%, an automatic gain control target of 100,000, a maximum ion target of 120 ms, and a resolution of 17,500. A dynamic exclusion value of 30 s was also used.

2.14 Bioinformatics for protein identification and quantification

All MS and MS/MS raw data files per sample were merged for protein identification and relative protein quantification into Proteome Discoverer vs 2.1 software (Thermo Scientific), enabling the database search by Mascot algorithm v. 2.4.2 (Matrix Science, UK) using the following criteria: UniProtKB protein database (*A. thaliana*, 89256 protein sequences, 09/2017) including the most common protein contaminants; carbamidomethylation of Cys and TMT6plex modification of lysine and peptide N-terminal as fixed modifications; oxidation of Met, deamidation of Asn and Gln, pyroglutamate formation of Gln as variable modifications. Peptide mass tolerance was set to ± 10 ppm and fragment mass tolerance to ± 0.02 Da. Proteolytic enzyme and maximum number of missed cleavages were set to trypsin and 2, respectively. Protein candidates assigned on the basis of at least two sequenced peptides and an individual Mascot Score grater or equal to 30 were considered confidently identified. For quantification, ratios of TMT reporter ion intensities in the MS/MS spectra from raw datasets were used to calculate fold changes between samples. Definitive peptide

assignment was always associated with manual spectra visualization and verification. Results were filtered to 1% false discovery rate.

3. Results and discussion

3.1 Overexpression of 14-3-3ε and 14-3-3ω isoforms

Transgenic *Arabidopsis* plants overexpressing either 14-3-3ω or 14-3-3ε isoforms were generated by *Agrobacterium*-mediated transformation. Three different lines for each construct (35S:14-3-3ω:YFP and 35S:14-3-3ε:YFP) were selected for kanamycin resistance; the presence of transgenic constructs was confirmed by PCR, using specific primers for the 35S promoter.

No phenotypical differences were observed between wild type- and 14-3-3-overexpressing plants. Total proteins extracted from T3 transgenic *Arabidopsis* lines were subjected to western blotting with anti-YFP and anti-14-3-3 antibodies, which recognize both 14-3-3 isoforms. Immunodecoration with anti-YFP antibodies showed that both isoforms were expressed to the same extent in the three lines (Fig. 1, panel A); on the other hand, immunodecoration with anti-14-3-3 antibodies demonstrated that they were highly expressed as compared to all the endogenous 14-3-3 isoforms (Fig. 1, panel B).

3.2 14-3-3ω and 14-3-3ε overexpression differentially affects H^+-ATPase activity

14-3-3 isoforms bind *in vitro* the H^+-ATPase with different affinities, thereby differently stimulating its phosphohydrolytic activity [36]. To verify whether a similar effect was also occurring *in vivo*, the H^+-ATPase activity of plasma membranes purified from 14-3-3ω- and 14-3-3ε-overexpressing lines was evaluated and compared to that of wild type plants. As shown in Fig. 2A, the overexpression of

266 the 14-3-3ω isoform brought about a marked (more than two-fold) stimulation of the H^+-ATPase
267 activity, whereas that of the 14-3-3ε was ineffective.
268 14-3-3 are cytosolic proteins that can localize at the plasma membrane depending on their
269 interaction with plasma membrane targets. The H^+-ATPase is the most abundant plasma membrane
270 14-3-3 client, thus variation of 14-3-3 isoforms bound to it can be evaluated by analyzing
271 corresponding 14-3-3 levels present in the plasma membrane fraction [48,49]. In order to confirm
272 that the higher activity of H^+-ATPase of 14-3-3ω-overexpressing plants was due to a greater ability
273 of 14-3-3ω to interact with the H^+-ATPase, plasma membrane fractions purified from 14-3-3ω- and
274 14-3-3ε-overexpressing plants were analyzed in parallel by western blotting with anti-YFP
275 antibodies. As shown in Fig. 2B, the 14-3-3ω isoform was markedly more associated with the
276 membrane fraction than the 14-3-3ε one, thus providing a rationale to the observed different H^+-
277 ATPase activity of corresponding samples. These results confirmed the idea that 14-3-3 proteins,
278 despite their high sequence identity and the same tridimensional structure, can display isoform-
279 specificity in the interaction with and in the regulation of their different targets.
280
281 ***3.3 Subcellular localization of 14-3-3ω and 14-3-3ε isoforms***
282 The subcellular localization of the 14-3-3ω and 14-3-3ε isoforms was also investigated by transient
283 expression in *Arabidopsis* protoplasts using 35S:14-3-3ω:YFP and 35S:14-3-3ε:YFP constructs,
284 respectively. Confocal laser scanning microscopy (Fig. 3) revealed a diffuse YFP fluorescence for both
285 constructs, in accordance with a cytosolic localization of both 14-3-3 isoforms. However, it is worth
286 noting that a significant fluorescence was also detectable at the plasma membrane level in 35S:14-
287 3-3ω:YFP-transformed protoplasts (Fig. 3, panel A), thus corroborating results obtained by western
288 blot analysis. The observed localization of 14-3-3ω at the plasma membrane was ascribed to its

association with the H^+-ATPase. In fact, it has been proposed that the 14-3-3 isoform subcellular localization is due to the interaction with specific targets [50].

Previous studies reported a nuclear localization of some 14-3-3 proteins [5; 50,51], which was not observed in our study. This can be explained considering that in those studies authors used specialized cells types, as trichomes and guard cells [5,50] in which peculiar 14-3-3 clients could be expressed [5,50].

3.4 14-3-3ω and 14-3-3ε overexpression improves cold stress tolerance

It has been recently demonstrated that exposure of *Arabidopsis* plants to 4 °C, for 18 h, stimulates plasma membrane H^+-ATPase activity, thus suggesting the involvement of this enzyme in the plant response to cold stress [52]. These data prompted us to investigate whether the overexpression of these specific two 14-3-3 isoforms, differentially regulating the H^+-ATPase activity, could affect the plant response to cold stress. To this purpose, 5-week old 14-3-3-overexpressing and WT plants were exposed to 4 °C, for 18 h. Although no major phenotypical differences between WT and transgenic plants were observed, analysis of electrolytes release and lipid peroxidation revealed a minor plasma membrane injury in 14-3-3-overexpressing plants [53,54]. As shown in Fig. 4A, values of relative conductance of both 14-3-3ω- and 14-3-3ε-overexpressing plants resulted significantly lower than that of WT plants. It is known that cold stress induces the accumulation of hydrogen peroxide and other reactive oxygen species (ROS) [55], which cause damage of macromolecules and cellular structures. The amount of hydrogen peroxide produced upon exposure to 4 °C, for 18 h, was then measured in WT and 14-3-3-overexpressing plants. As shown in Fig. 4B, cold stress induced the accumulation of hydrogen peroxide to a markedly higher level in WT plants than in both 14-3-3ω- and 14-3-3ε-overexpressing counterparts. Accordingly, malondialdehyde (MDA) production resulted consistently lower in both 14-3-3ω- and 14-3-3ε-overexpressing lines, as compared to WT

313 plants, indicating that a reduced membrane lipid peroxidation was occurring in transgenic plants
314 (Fig. 4, panel C).
315 It has been reported that plants can accumulate intracellular free proline in response to low
316 temperature [56,57]; this phenomenon has been related to a protective role of this amino acid
317 toward proteins and membrane structures [58]. In particular, proline accumulation during abiotic
318 stresses was demonstrated to counteract oxidative stress [59], and was possibly associated with an
319 augmented regulation of the activity of the corresponding biosynthetic enzymes [60].
320 Since 14-3-3-overexpressing *Arabidopsis* plants showed reduced injuries upon cold stress, we
321 investigated whether these plants accumulated increased amounts of free proline as compared to
322 WT. As shown in Fig. 4D, no difference in free proline content was observed in the absence of cold
323 stress, while, upon low temperature challenge, free proline levels increased differentially in all
324 plants. Transgenic plants exhibited an increased proline content as compared to WT, and this effect
325 was significantly more relevant for 14-3-3ε-overexpressing plants.
326
327 ***3.5 Proteomic analysis of cold stressed 14-3-3ε-overexpressing Arabidopsis plants***
328 In order to get a deeper insight about the effect of the over-expression of 14-3-3 isoforms
329 concerning the response to cold stress of *Arabidopsis* plants, a TMT-based proteomic investigation
330 was undertaken. Since over-expression of 14-3-3ε or ω isoforms brought about the same effect on
331 the induction of tolerance to cold, differential proteomics was restricted to plants transformed with
332 the 14-3-3ε (GRF10) isoform. The rationale for this choice was that, as reported above, the over-
333 expression of the 14-3-3ω determined a stimulation of the plasma membrane H^+-ATPase, an effect
334 that could *per se* alter gene expression, therefore potentially overlapping with that brought about
335 by cold stress. TMT-label based proteomics allowed the identification and quantification of 3045
336 unique proteins from *Arabidopsis* leaves. Only proteins with an abundance variation ≥ 2 or ≤ 0.5-

fold were considered as differentially represented. As expected, 14-3-3ε-overexpressing plants grown under control conditions (23 °C) showed an increase (three-fold) of 14-3-3ε levels in leaves, when compared to WT counterparts (Table 1 and Fig. 5). However, over-expression of the 14-3-3ε isoform *per se* did not profoundly affect the protein repertoire of *Arabidopsis* leaves (Table 1 and Fig. 5); in fact, under control conditions (23 °C), the unique over-represented protein was β-glucosidase 23, whereas 4 proteins were down-represented, namely late embryogenesis abundant protein 7 (LEA7), temperature-induced 65 kDa protein (LTI65), stress-induced protein KIN1 (KIN1) and subtilisin-like protease SBT5.3 (AIR3) (Table 1 and Fig. 5). As expected, also 14-3-3ε-overexpressing plants when exposed to cold stress (4 °C, for 18 h) showed a three-fold increase of 14-3-3ε levels, as compared to WT counterparts, while no other quantitative protein variation was detected.

A more pronounced effect of cold stress on the differential representation of proteins in 14-3-3ε-overexpressing plants was revealed when a pairwise comparison between 14-3-3ε-overexpressing plants at 4 °C *vs* 14-3-3ε-overexpressing plants at 23 °C as compared to WT plants at 4 °C *vs* WT plants at 23 °C was considered (Table 1 and Fig. 5). In the whole, 30 protein species resulted as differentially abundant. Among these, only Dormancy associated protein 1 (DRM1) contains a canonical 14-3-3-binding motif, thus suggesting that the effect of 14-3-3ε overexpression was not due to a direct association with differential represented proteins and regulation of their stability [24,61]. When the differentially represented species were subjected to STRING analysis, an interaction network linking together most (21 over 30, plus 14-3-3ε) components was observed (Fig. 6A), suggesting the occurrence of a complex mechanism associated with adaptation to cold stress in 14-3-3ε-overexpressing plants. Functional categorization according to Gene Ontology annotation and literature data revealed that most proteins grouped in the functional class of stress response, particularly response to cold and oxidative stresses.

When over-represented proteins were considered, proteomic analysis of 14-3-3ε-overexpressing plants demonstrated 15 components showing increased levels upon cold stress, whereas in WT plants subjected to cold stress only 8 proteins out of those 15 showed a corresponding increase in abundance. A Venn diagram showing the partial overlap in the response to cold of 14-3-3ε-overexpressing and WT plants is reported in Fig. 6B. Within the above-mentioned over-represented proteins, 8 have already been reported to be related to response to stress. Among these, adenylysulfate reductase 2 (APR2), calmodulin like protein 10 (CML10), low temperature-induced 78 kDa protein (RD29A), vegetative storage protein 2 (VSP2) were increased exclusively in 14-3-3ε-overexpressing plants, whereas gluthatione S-transferase U25 (GSTU25), peptide methionine sulfoxide reductase B3 (MSRB3), galactinol synthase 3 (GOLS3) and peptidyl-prolyl *cis-trans* isomerase FKBP65 (FKBP65) were increased in both 14-3-3ε-overexpressing and WT plants. Among the first group, RD29A was already proved to be induced by various abiotic stresses, including salt, drought and cold [62], and by ABA [63]. Previous studies also demonstrated that *RD29A* gene transcription was increased by low temperatures in response to elevated cytosolic Ca^{2+} concentration values [64], as a part of the CBF pathway of cold acclimation [65]. In the context of Ca^{2+} metabolism, worth mentioning is also the observed over-representation of CML10, which is a member of the calmodulin-like protein family (CMLs), a novel and less studied class of Ca^{2+} sensors [66]. It is well known that Ca^{2+} signaling is involved in the response to oxidative stress [67]; recently, it has been reported that *cml10 Arabidopsis* knock-down mutants are more sensitive to oxidative stress, thus suggesting that CML10 can modulate ROS levels by regulating ascorbic acid synthesis through interaction with phosphomannomutase [66]. VSP2 is a member of the VSPs class of nitrogen-accumulating proteins that accomplish a nutritional role during plant development; these proteins were also demonstrated as being involved in the response to various stresses, such as wounding [68] and oxidative insult [69]. Finally, APR2 is involved in the sulfate reduction to sulfite

385 for the assimilation of sulfur into cysteine, methionine and other essential compounds. Thus, it can
386 control sulfur flux into glutathione, therefore contributing to cell redox homeostasis, particularly
387 under oxidative stress conditions [70]. Its over-representation appears to be fairly correlated to the
388 observed increase of glutathione S-transferase U25 levels (see below) to meet the augmented
389 demand of plant for GSH under cold stress conditions. CML10, APR2, LTI78 and 14-3-3ε appear as
390 key knots in the interaction network reported in Fig. 6A, which link together different subnetworks.
391 Among over-represented proteins in 14-3-3ε-overexpressing and WT plants after cold stress, worth
392 mentioning is GSTU25, which belongs to the large GSTs family of enzymes ensuring detoxification
393 of xenobiotics, and function as GSH-dependent peroxidases, catalyzing the reduction of organic
394 hydroperoxides to monohydroxy alcohols, thereby limiting corresponding oxidative damage [71].
395 MSRB3 is an enzyme that catalyzes the reduction of methionine sulfoxide to methionine in oxidized
396 proteins. It plays a protective role by restoring activity to proteins that have been inactivated by
397 methionine oxidation, and it has been reported to be involved in cold acclimation [72]. FKBP65
398 belongs to the large family of cyclophilins that assist isomerization of proline imidic peptide bond,
399 which is a rate-limiting step in protein folding [73]. They have been reported being induced in
400 response to various abiotic and biotic stresses [74,75]. Interestingly, it has been demonstrated that
401 the heterologous expression of a wheat cyclophilin TaCypA-1 confers thermotolerance to
402 *Escherichia coli* [76]. Finally, GOLS3 catalyzes the first step in the synthesis of the raffinose family of
403 oligosaccharides from UDP-glucose. This enzyme very likely plays a pivotal role in conferring
404 tolerance to cold, since it has been demonstrated that cold-stressed *Arabidopsis* plants accumulate
405 high amounts of galactinol and raffinose [77]. Overall, above-reported data clearly indicated that
406 both 14-3-3ε-overexpressing and WT plants after cold stress elicited the expression of genes
407 specifically deputed to induction of tolerance to cold and oxidative damage managing, but also

demonstrated that in the transgenic plants a more various and robust expression of protective genes took place.

Proteomic analysis also revealed 15 proteins whose levels decreased upon cold challenge; among that, 8 varied only in WT plants, while 6 changed their levels both in 14-3-3ε-overexpressing and WT plants, and 1 only in 14-3-3ε-overexpressing plants (Fig. 6B). Various of these down-represented proteins have already been related to stress response, namely LEA7, LTI65, KIN1, non-specific lipid-transfer protein 3 and 4 (LTP3 and 4), endochitinase CHI (CHI), DNA repair RAD52-like protein 1 (RAD52-1) and dormancy-associated protein 1 (DRM1). The first 6 proteins were specifically down-represented in WT plants after cold stress, suggesting a selective repression of down-regulation of corresponding genes in 14-3-3ε-over-expressing plants, probably as result of the existence of compensative mechanisms against this temperature insult in these transgenic plants. LTI65, LEA7, KIN1, LTP3 and LTP4 appear as interconnected in Fig. 6A. On the other hand, the remaining two proteins (RAD52-1 and DRM1) were down-represented in WT and 14-3-3ε-overexpressing plants.

Considering proteins specifically down-represented exclusively in WT plants, worth mentioning is LEA7, a member of the LEA protein family, whose primary function is to confer desiccation tolerance during seed maturation but that is also involved in the tolerance to stress. Recently, LEA7 has been demonstrated to preserve *in vitro* enzyme activity upon freezing [78]. In WT plants, abundance of LEA7 was greatly reduced by cold, whereas it was statistically unaffected in 14-3-3ε-overexpressing plants. A quite similar WT plant-specific down-representation was observed for: i) LTI65, a protein involved in the response to different environmental stresses, including cold [79]; ii) LTP3 and LTP4, which belong to a protein family participating to cutin assembly also in response to biotic stress [80]; although information about their involvement in the response to abiotic stress is still very poor, LTP3 overexpression resulted in constitutively enhanced tolerance to freezing [81]; iii) KIN1, a protein induced by osmotic [82], ABA and cold stresses [83,84], which also shows a

peculiar trend of regulation. In fact, whereas low temperature in wild-type plants brought about a decrease of its abundance, an increase of about the same extent was determined in 14-3-3ε-overexpressing plants. Regarding stress-related proteins down-represented in both WT and 14-3-3ε–overexpressing plants, DNA repair RAD52-like protein 1 (RAD52-1) is a plant-specific single-stranded DNA-binding protein, which is involved in double-stranded DNA break repair [85], whereas dormancy-associated protein 1 (DRM1) is a member of the DRM1/ARP protein family that is considered a genetic marker of dormant meristematic tissues. On the basis of their structure similarity with LEA, GRAS and HSP proteins, increasing evidence suggests that this class of proteins is also involved in the plant response to stress [86]. On the whole, data on down-represented proteins confirmed those concerning over-represented ones: 14-3-3ε-over-expressing plants were less sensitive than wild type ones to the reduction in abundance of protective proteins upon cold stress conditions.

Conclusion

In this study, two 14-3-3 isoforms, namely ω and ε, were independently overexpressed in *Arabidopsis thaliana* plants. 14-3-3ω overexpression brought about a marked stimulation of the H^+-ATPase activity, whereas that of the 14-3-3ε isoform was ineffective. Overexpression of both isoforms produced plants more tolerant to low temperature stress which showed a reduced ion leakage, H_2O_2 production and membrane lipid peroxidation, as well as an increased production of free proline. A dedicated TMT-based proteomic analysis of 14-3-3ε-overexpressing plants provided useful information to rationalize the physiological properties of transformed plants. In fact, it demonstrated that different proteins involved in the plant response to cold or oxidative stress were over-represented or not down-represented in 14-3-3ε-overexpressing plants. Since among the over-represented proteins no anti-oxidant enzymes (superoxide dismutase, catalase, ascorbic

peroxidase) were found, the reduced oxidative stress detected in transgenic plants could be explained by post-translation regulation of anti-oxidant enzymatic activity. Alternatively, protection from oxidative stress could be due to the increase in proline content of transformed plants; in fact, it has been reported that proline accumulation during abiotic stresses can counteract oxidative stress [59]. Proteomic analysis also suggested that the augmented levels of proline observed in challenged 14-3-3ε-overexpressing plants were not associated with representation changes of the corresponding biosynthetic enzymes, but rather to a possible positive regulation of their enzymatic activities [60].

Finally, proteomic data revealed that levels of two proteins participating to the COR pathway of response to cold stress, namely KIN1 and RD29A, were over-represented in cold-stressed 14-3-3ε-overexpressing plants, with respect to WT, suggesting a role for this isoform as a positive regulator of cold adaptation. In this context, Liu and coworkers already reported 14-3-3λ as a negative regulator of freezing tolerance, which acts according to a mechanism involving its migration into the nucleus [24], where it interacts with and destabilizes CBF proteins of the COR pathway. Reasons for the opposite action of 14-3-3λ and 14-3-3ε as negative and positive regulators, respectively, need further investigations, but it is worth remarking that subcellular localization, client interaction and regulatory function properties in plants are 14-3-3 isoform-specific [50]. In this context, the different subcellular districts (cytosol for 14-3-3ε and nucleus for 14-3-3λ) in which these proteins possibly elicit their regulatory action on cold adaptation and freezing tolerance, respectively, should suggest that they likely act according to independent molecular processes. In conclusion, this and above-mentioned study [24] support the notion that cold adaptation and freezing tolerance are not overlapping complex responses, which involve multiple mechanisms in which various 14-3-3 isoforms may play a different regulatory role.

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Table 1. Differentially represented proteins identified in this study. Reported are protein accession, protein name, gene name, sum posterior error probability (PEP) score, sequence coverage (%), number of identified peptides, peptide spectrum matches (PSMs), number of unique identified peptides and Mascot score.

Accession	Description	Gene name	Sum PEP score	Coverage (%)	Peptides	PSMs	Unique Peptides	Mascot score
P48347	14-3-3-like protein GF14 epsilon	GRF10	297.363	75	20	298	17	10493
P92981	5'-adenylylsulfate reductase 2	APR2	63.134	19	6	24	6	840
Q9SR37	Beta-glucosidase 23	BGLU23	137.267	40	15	55	13	1555
P30187	Calmodulin-like protein 10	CML10	10.568	10	2	3	2	103
P16972	Ferredoxin-2, chloroplastic	FD2	25.436	34	2	28	1	880
O80518	Galactinol synthase 3	GOLS3	11.723	9	3	3	3	86
Q9SHH7	Glutathione S-transferase U25	GSTU25	5.749	7	2	7	1	128
Q06738	Low-temperature-induced 78 kDa protein	RD29A	252.727	49	26	95	25	3103
Q9XFM6	Membrane steroid-binding protein 1	MSBP1	44.836	30	3	11	3	479
Q9M0Z6	Peptide methionine sulfoxide reductase B3	MSRB3	10.9	14	2	7	1	202
Q9FJL3	Peptidyl-prolyl cis-trans isomerase FKBP65	FKBP65	9.671	5	3	4	1	98
Q9C6B9	Phosphoethanolamine N-methyltransferase 3	NMT3	41.802	19	9	16	6	386
Q8L970	Probable prolyl 4-hydroxylase 7	P4H7	9.181	7	2	3	2	93
P54121	Protein AIG2 A	AIG2A	10.119	12	2	3	2	77
Q9CAB6	Ubiquitin-conjugating enzyme E2 variant 1B	UEV1B	7.773	12	2	10	1	179
O82122	Vegetative storage protein 2	VSP2	69.04	26	7	42	2	1631
P0CAN7	V-type proton ATPase subunit E3	VHA-E3	69.197	26	7	41	1	1481
P49078	Asparagine synthetase [glutamine-hydrolyzing] 1	ASN1	105.677	22	9	33	7	1174
Q9SCV8	Beta-galactosidase 4	BGAL4	28.529	8	5	7	4	181
Q9FVV7	DNA repair RAD52-like protein 1, mitochondrial	RAD52-1	10.238	18	2	2	2	67
B9DGG8	Dormancy-associated protein 1	DRM1	22.037	43	4	6	4	177
O24603	Endochitinase CHI	CHI	24.311	12	2	4	2	180
Q8LEJ6	Fasciclin-like arabinogalactan protein 11	FLA11	19.585	15	2	5	2	192

Q96270	Late embryogenesis abundant protein 7	LEA7	83.759	50	9	29	9	891
Q04980	Low-temperature-induced 65 kDa protein	LTI65	120.183	33	13	44	12	1625
Q9LLR7	Non-specific lipid-transfer protein 3	LTP3	71.396	54	5	62	5	2106
Q9LLR6	Non-specific lipid-transfer protein 4	LTP4	66.93	57	6	57	5	1950
Q9FPH3	Probable low-specificity L-threonine aldolase 2	THA2	20.444	12	3	4	3	116
P18612	Stress-induced protein KIN1	KIN1	80.511	74	5	35	4	1458
Q9ZSP5	Subtilisin-like protease SBT5.3	AIR3	8.648	2	2	3	2	76
Q8LBZ7	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit 1	SDH2-1	36.262	25	5	14	1	465
P23586	Sugar transport protein 1	STP1	37.057	10	4	10	4	451
Q8GXB1	UPF0548 protein At2g17695	At2g17695	21.21	23	3	4	3	109

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Figure legends

Fig. 1. 14-3-3ω and 14-3-3ε levels in Arabidopsis overexpressing lines

20 µg of total proteins extracted from wild type and three T3 transgenic Arabidopsis lines overexpressing 14-3-3ω-YFP and 14-3-3ε-YFP were subjected to SDS-PAGE, blotted onto PVDF membrane and immunodecorated with anti-YFP (panel A) or anti-14-3-3 (panel B) antibodies. Actin was used as loading control.

Fig. 2. Effect of 14-3-3ω and 14-3-3ε overexpression on the H^+-ATPase activity

A. Phosphohydrolytic activity of plasma membrane fractions (50 µg of proteins) purified from 5-week old wild type and 14-3-3-overexpressing Arabidopsis plants.

B. Association of 14-3-3ω and 14-3-3ε with the plasma membrane: 20 µg of plasma membrane proteins were run in SDS-PAGE, blotted onto PVDF membrane and immunodecorated with anti-YFP antibodies.

Experiments were carried out using plasma membrane preparations obtained by pooling 5-week old Arabidopsis plants of the three T3 homozygous lines. Ponceau staining of immunoblotted membrane was used as loading control

Fig. 3. Subcellular localization of 14-3-3ω and 14-3-3ε isoforms. Representative confocal single sections of *Arabidopsis* mesophyll protoplasts expressing 14-3-3ω-YFP (A) and 14-3-3ε-YFP (B) fusion. CellMask Orange was used for plasma membrane staining. YFP fluorescence is shown in green, chlorophyll autofluorescence is shown in red, CellMask Orange fluorescence is shown in yellow. Scale bar, 10 µm.

Fig. 4. Effect of 14-3-3ω and 14-3-3ε overexpression on cold tolerance

Wild type and 14-3-3-overexpressing plants were exposed to 4 °C, for 18 h, and leaves were used to evaluate the ion leakage release (panel A), hydrogen peroxide production (panel B), lipid peroxidation, measuring MDA production (panel C), and free proline content (panel D). Experiments were carried out with preparations obtained by pooling 5-week old *Arabidopsis* plants of the three T3 homozygous lines for each transformation. Black bars, control plants; white bars, plants exposed to 4 °C. Data are the means ± standard error for four independent experiments.

Fig. 5. Proteins showing quantitative changes in the comparison of 14-3-3ε-overexpressing plants at 4 °C *vs* 14-3-3ε-overexpressing plants at 23 °C (14-3-3ε cold *vs* 14-3-3ε control), and WT plants at 4 °C *vs* WT plants at 23 °C (WT cold *vs* WT control).

Reported are differentially represented proteins shown in Table 1, which are symbolized using corresponding gene names. This figure also includes 14-3-3ε and β-glucosidase that were over-represented in the 14-3-3ε control *vs* WT control comparison.

Fig. 6. Bioinformatic and correlation analysis of results from proteomic comparisons of 14-3-3ε-overexpressing WT plants exposed to cold stress.

A. STRING analysis of differentially represented proteins reported in this study, as obtained using default software setting parameters and adding more nodes to the network output resulting from components described in Table 1 (Panel A). Proteins are symbolized according to their gene names (see Table 1) and those differentially represented are boxed. GRF10 (14-3-3ε) is marked by an arrow.

B. Venn diagram showing unique and common components deriving from the comparison of results from 14-3-3ε cold *vs* 14-3-3ε control with respect to that from WT cold *vs* WT control.

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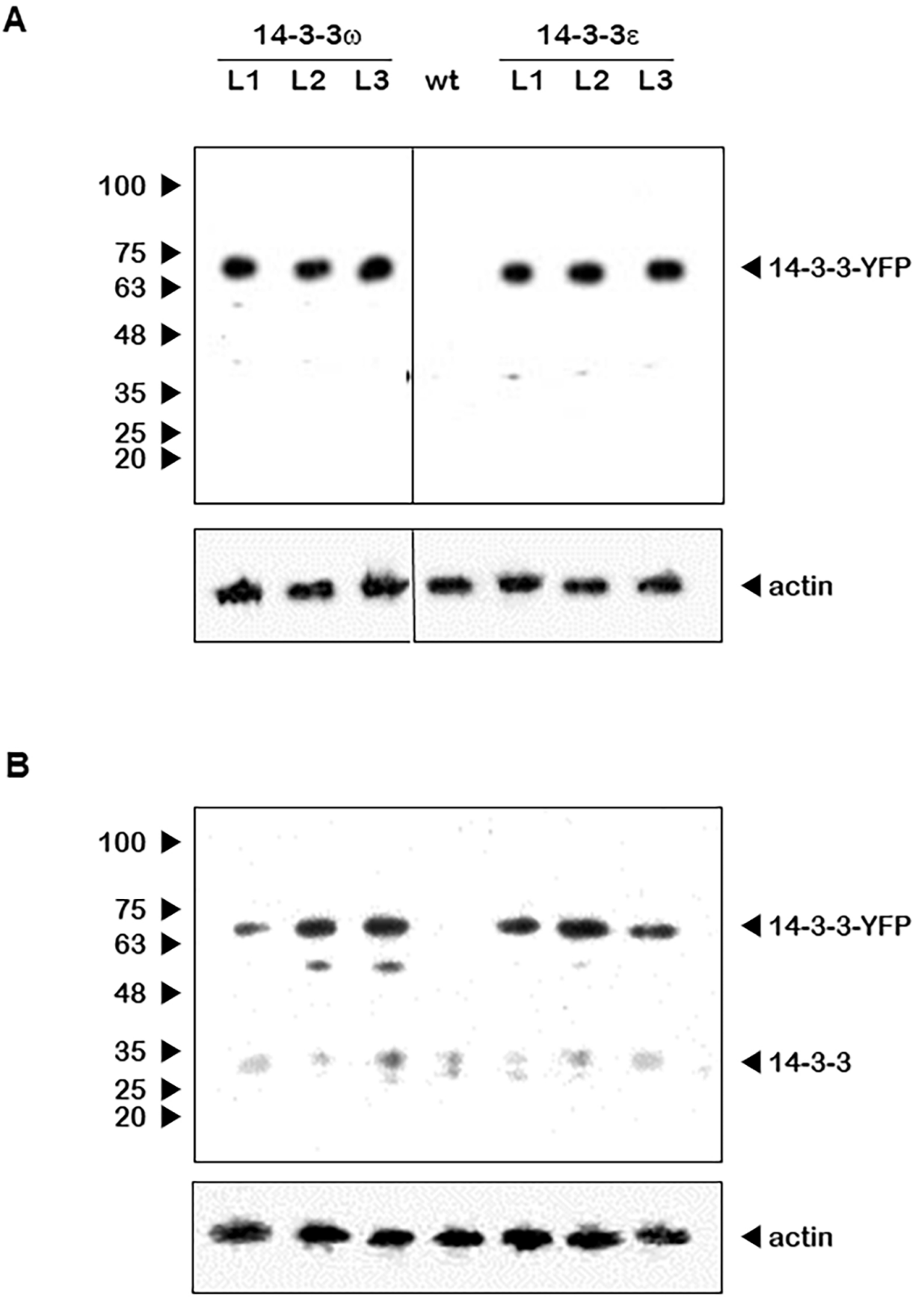
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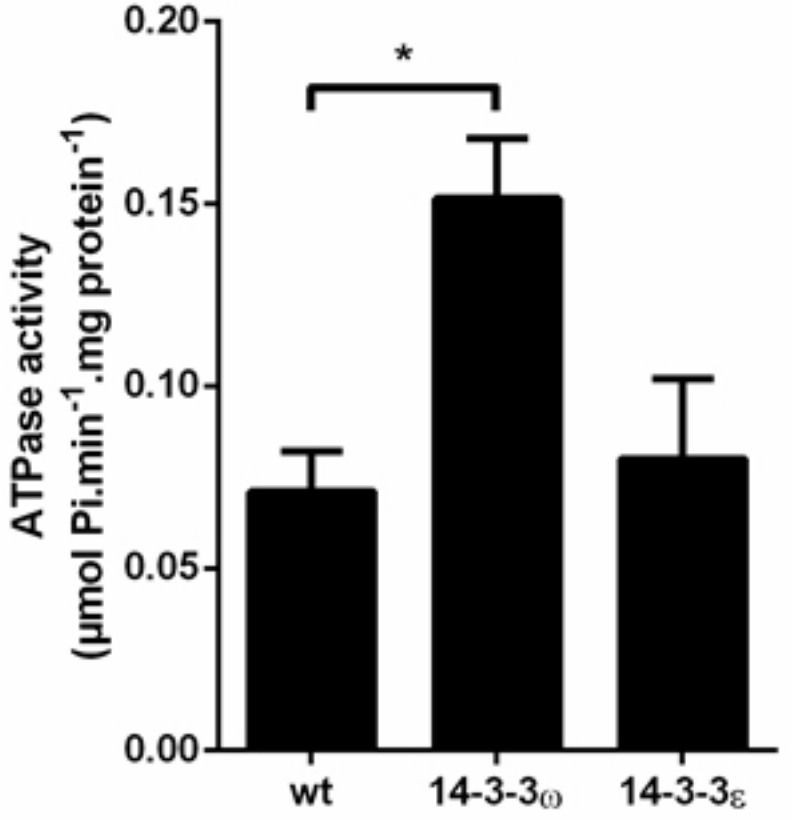
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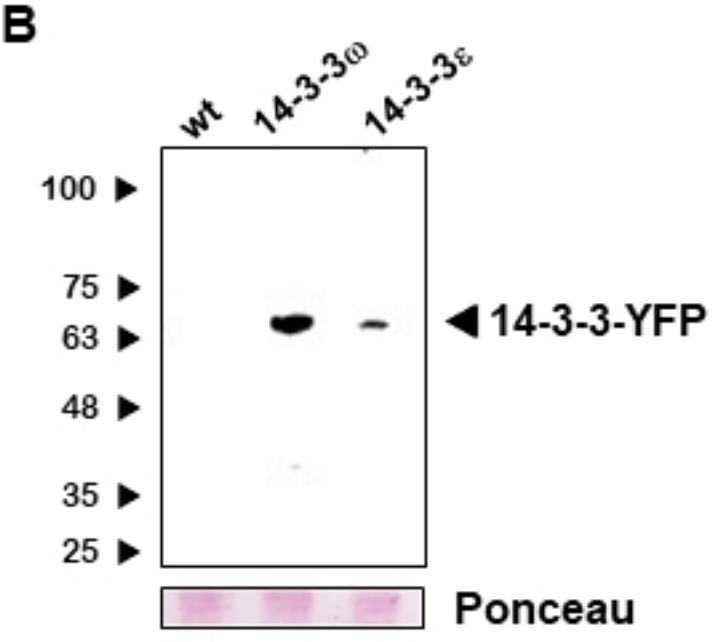
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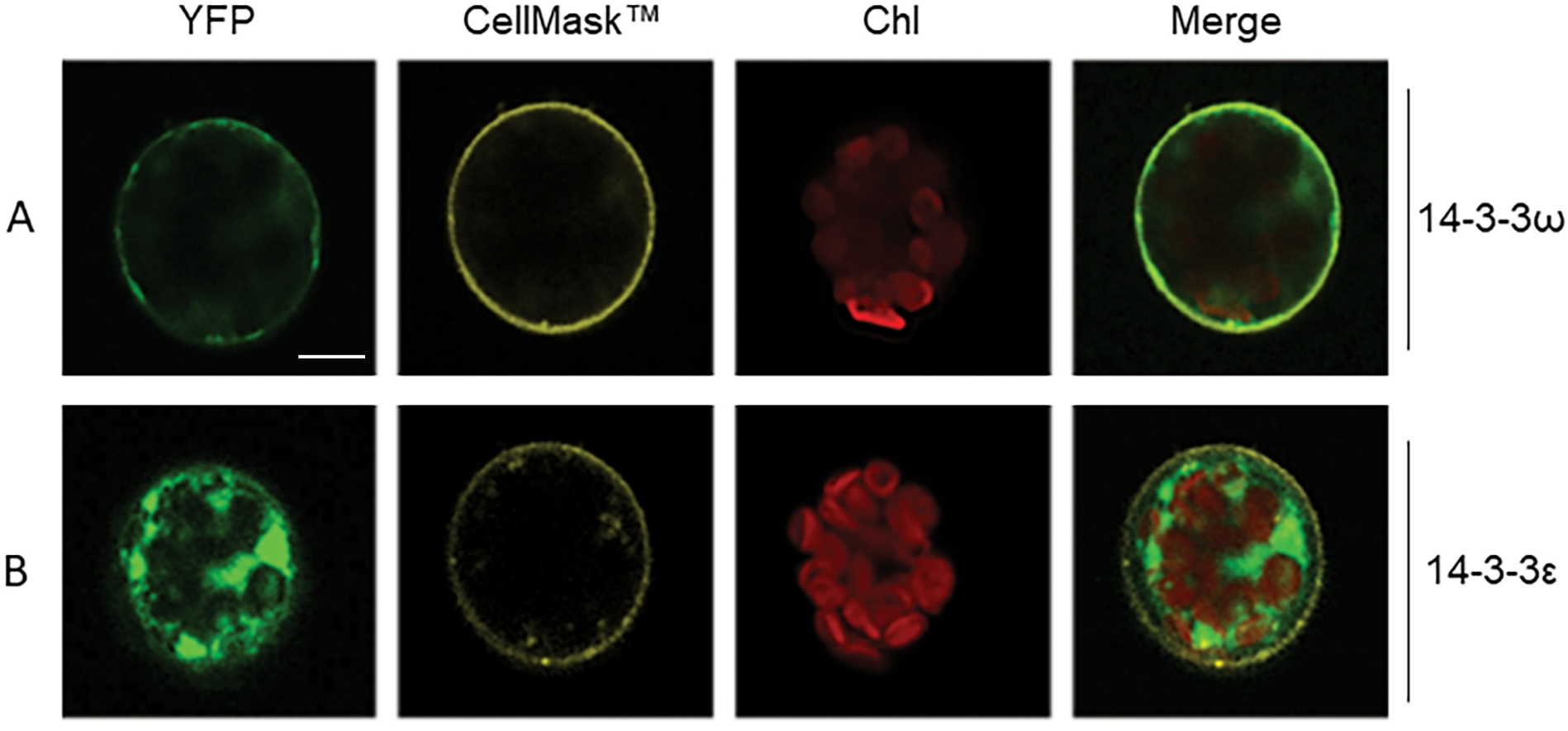
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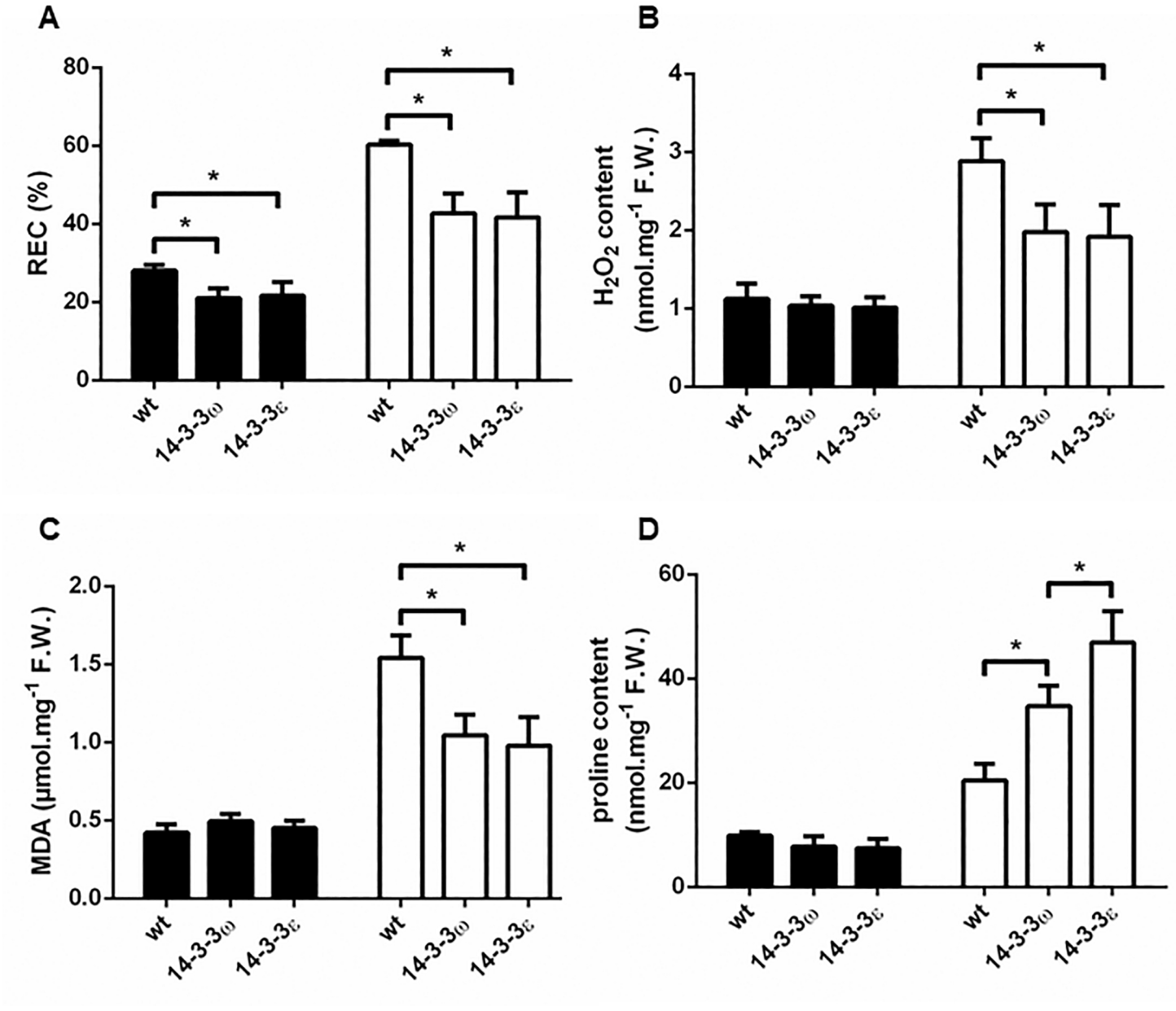
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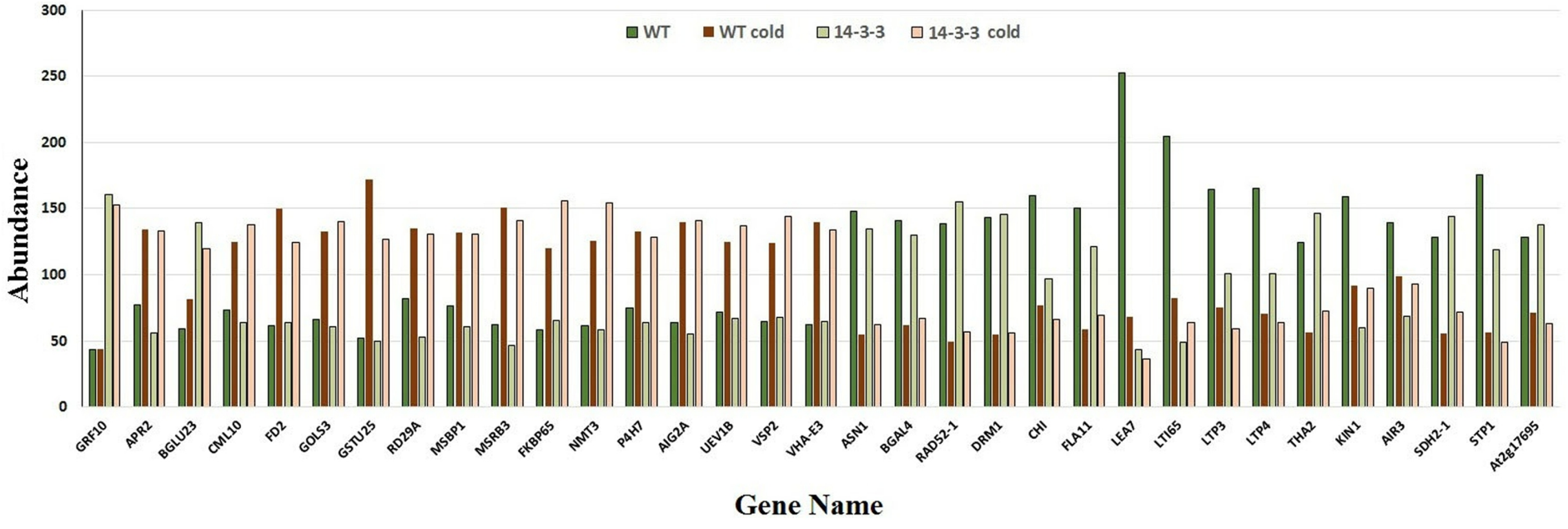
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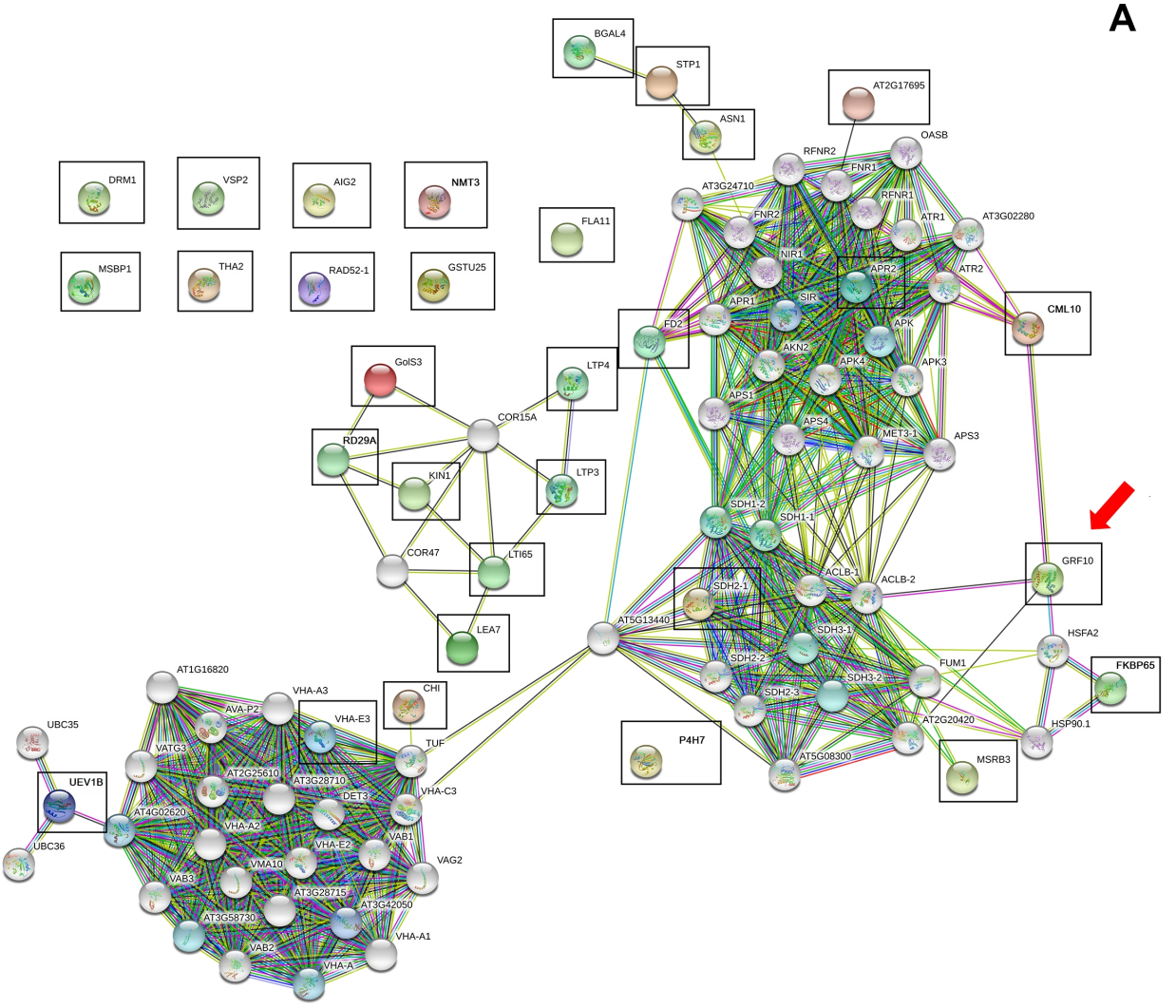
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SDH2-2
FUM1
FKBP65
SDH3-2
SDH2-3
AT2G20420
HSP90.1
P4H7
AT5G08300
MSRB3
LEA7
AT1G16820
VHA-A3
CHI
AVA-P2
VHA-E3
UBC35
TUF
VATG3
UEV1B
AT2G25610
AT3G28710
DET3
VHA-C3
AT4G02620
VHA-A2
VHA-E2
VAB1
UBC36
VAG2
VMA10
VAB3
AT3G28715
AT3G42050
AT3G58730
VHA-A1
VAB2
VHA-A
DRM1
VSP2
AIG2
NMT3
FLA11
MSBP1
THA2
RAD52-1
GSTU25
B
14-3-3 ε cold vs 14-3-3 ε control
7
1
8
6
8
WT cold vs WT control

LEGEND TO SUPPLEMENTARY TABLES

Supplementary Table S1.

Identification details of the differentially represented proteins reported in this study. Reported are the rough identification and quantification results from mass spectrometry, as well as the corresponding identification parameters. Reported are the information on protein false discovery rate (FDR) confidence, protein accession, description, exp. *q*-value, sum posterior error probability (PEP) score, sequence coverage (%), number of identified peptides, ~~P~~peptide ~~S~~spectrum ~~M~~matches (PSMs), number of identified unique peptides, protein groups, number of amino acids, molecular mass, pI, Found in file, Found in sample, modification(s), ~~Abundance~~ abundance ~~Ratio~~ratio, ~~Abundance~~ abundance (~~Grouped~~grouped), ~~A~~abundance (~~G~~grouped) ~~S~~standard ~~E~~error (%), ~~Abundance~~ abundance (~~Scaled~~scaled), ~~-~~emPAI, Razor ~~Peptides~~peptides, and Mascot identification score values ~~and peptides~~. Specific information on the identified peptides for each protein are also reported, including identification confidence, sequence, modification, Qvality PEP, Qvality *q*-value, protein groups, proteins, PSMs, master protein accession, position, missed cleavage, theor. MH^+, Found in File, Found in sample, ~~Abundance~~ abundance ~~Ratio~~ratio, ~~Abundance~~ abundance (~~Grouped~~grouped), ~~Abundance~~ abundance (~~Scaled~~scaled), Quan info, ion score, Mascot confidence, Percolator *q*-values and Percolator PEP Mascot.