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Involvement of Phosphatidylinositol 3-kinase in the regulation of proline catabolism in Arabidopsis thaliana

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INTRODUCTION

As sessile organisms, plants need to cope with adverse environmental stresses. Abiotic constraints such as drought and salinity have a major impact on plant development and crop productivity (Zhu, 2002). A common feature of drought and salt stress is the lower availability of water, due to decrease of soil water potential. In addition, salt generates an ionic stress due to the presence of Na+ and Cl−. Perception of drought and salt constraints triggers complex signaling networks, which then induce the adaptive response of plants. Among these networks, various molecular components are involved, including phytohormones, protein kinases and phosphatases, and second messengers like Ca2+, ROS, and lipid signaling elements (Munnik and Vermeer, 2010; Huang et al., 2012; Delein et al., 2014; Golldack et al., 2014).

Phospholipids are important structural components of cellular membranes but can also play an essential role in the adaptation of plants to abiotic stress (Munnik and Testerink, 2009; Xue et al., 2009; Munnik and Vermeer, 2010; McLoughlin and Testerink, 2013). They are modified by enzymes such as phospholipase C (PLC) and D (PLD), and by lipid-kinases, such as diacylglycerol kinase (DGK), PA kinase and various phosphoinositide kinases (Meijer and Munnik, 2003). These modifications produce important second messengers that regulate various plant responses.

Phosphatidylinositol 3-kinase (PI3K) phosphorylates the D-3 position of the inositol ring of phosphoinositides. In mammals, three distinct PI3K classes (I-III) can be distinguished, differing in gene structure, enzyme regulation, and substrate preference. Class III PI3Ks are homologous to the yeast VPS34, which uses PI as a sole substrate to produce PI3P (Backer, 2008). VPS34 promotes membrane fusion and vesicle trafficking by recruiting PI3P-binding proteins to membranes. VPS34 is associated with different proteins, forming distinct protein complexes, including the regulation of mTORC1 (Target of rapamycin complex 1) that monitors the nutritional status of the cell (Backer, 2008; Ktistakis et al., 2012; Robaglia et al., 2012).

Higher plants only contain VPS34-like PI3Ks (Lee et al., 2010). In Arabidopsis, PI3K activity is encoded by a single gene (At1g60490), which is important for pollen development...
At the subcellular level, LY294002 blocks endocytosis and vacuolar trafficking and inhibits auxin-mediated ROS generation (Servet et al., 2012). ProDH is considered as the main isoform, ProDH1 being weakly expressed (Kiyosue et al., 1996; Funck et al., 2010). Under either salt or drought stress, ProDH1 expression is repressed allowing proline accumulation (Funck et al., 2010). On the opposite, when stress is relieved, ProDH1 expression is triggered leading to proline degradation in mitochondria (Kiyosue et al., 1996; Verbruggen et al., 1996).

Proline accumulation in response to water stress is not only important for osmotic adjustment, but also as scavenger for reactive oxygen species (ROS) and molecular chaperone to stabilize proteins, antioxidant enzymes and membrane structures (Szabados and Savouré, 2010; Liang et al., 2013). Proline is also considered as a source of energy, which may be important upon stress recovery (Szabados and Savouré, 2010; Liang et al., 2013; Kavi Kishor and Sreenivasulu, 2014).

As proline accumulation and degradation result from a tight regulation of its metabolism, deciphering the signaling networks involved is of prime importance. Activation of proline biosynthesis is linked to both ABA mediated-signal transduction (Strizhov et al., 1997; Abrahám et al., 2003) and ABA-independent signaling (Savouré et al., 1997; Sharma and Verslues, 2010). P5CS1 expression was also shown to be positively regulated by ROS, acting as intermediate in ABA-mediated proline accumulation, while ProDH activity was repressed (Yang et al., 2009).

In Arabidopsis, lipid signaling components are involved in the regulation of P5CS1 expression. Under normal growth condition, PLD negatively regulates P5CS1 expression, preventing proline accumulation (Thierry et al., 2004). Upon ionic but not osmotic stress, PLC triggers P5CS1 expression leading to proline accumulation (Parre et al., 2007). P5CS1 up-regulation by PLC involves Ca2+ as a second messenger, which acts as a molecular switch to trigger downstream signaling events (Parre et al., 2007). Expression of both ProDH genes is regulated by bZIP transcription factors (Weltmeier et al., 2006; Hanson et al., 2008). After dark treatment or in response to hypoosmolarity stress, ProDH1 expression is induced by the heterodimer bZIP53/bZIP10 which recognizes the ACTCAT regulating sequence in ProDH1 promoter.
with either 200 mM NaCl or 400 mM mannitol and with the same as a control. Seedlings were thereafter transferred for 3 h or 24 h LY294002 dissolved in DMSO or with the same amount of DMSO

1 h in 0.5 µL

PHOSPHOLIPIDS ANALYSIS

For practical reasons this experiment was performed on 3- to 5-days-old seedlings into a 2 mL Eppendorf tube containing 200 µL of 2.5 mM MES/KOH buffer (pH 5.7) and 1 mM KCl. In order to label phospholipids, 10 µCi of 32P-inorganic phosphate were added in each tube and incubated overnight (Munnik and Zarza, 2013). Either 100 µM LY294002 or the same amount of DMSO for the control were then added for 1 h of pre-incubation. Then a volume of 2.5 mM MES/KOH buffer (pH 5.7), 1 mM KCl buffer with 400 mM NaCl was added into the tube to reach a final concentration of 200 mM NaCl. For control condition, an equivalent volume of MES/KCl buffer was added. Treatments were stopped just after the addition of NaCl (0 h), or after 30 min or 3 h, by adding perchorlic acid (5% w/v, final concentration), and after 10 min shaking the total solvent was removed. To extract lipids from the seedlings, 400 µL CHCl3/MeOH/HCl (50/100/1, v/v/v) was added and the mix was vortexed for 10 min. To induce the separation of two phases, 400 µL CHCl3 and 200 µL 0.9% (w/v) NaCl were added, vortexed 10 s and then centrifuged for 1 min at 10,000 g. The organic lower phase was transferred to a new tube containing 400 µL CHCl3/MeOH/HCl HCl (3/48/47, v/v/v). After shaking and centrifugation, the upper phase was removed, and 20 µL isopropanol was added to the purified organic phase, which was then dried down in a vacuum centrifuge at 50°C. The residue was dissolved in 100 µL CHCl3.

Phospholipids were separated as previously described (Munnik et al., 1994, 1995; Munnik and Zarza, 2013) by thin-layer chromatography (TLC) using heat-activated silica gel plates impregnated with a solution of 1% K-oxalate, 2 mM EDTA in MeOH/H2O (2/3, v/v) and chromatographed with an alkaline solvent CHCl3/MeOH/NH4OH/H2O (90/70/4/16, v/v/v/v). Radiolabeled phospholipids were visualized and quantified using a phospholmager.

In order to separate PI3P and PI4P, spots corresponding to the PIP pool was scraped off from the TLC plate and deacylated with 800 µL of mono-methylamine reagent (25% mono-methylamine/MeOH/n-ButOH (42.8/45.7/11.5, v/v/v) at 53°C for 30 min as described in (Munnik et al., 1994, 1996; Munnik, 2013). Samples were centrifuged at 10,000 g for 2 min and the supernatant was collected and dried under a N2 stream for 30 min and then by rotary evaporation. To remove the fatty-acyl groups, samples were dissolved in 500 µL H2O and extracted twice with 600 µL n-ButOH/petroleum ether (40–60°) ethyl formate (20/40, v/v/v). The aqueous lower phase that contains glycerophosphoinositides (GroPlnSP) was dried by rotary evaporation, dissolved in 500 µL H2O and filtered (0.22 µm).

GroPlnSP3P and GroPlnSP4P were separated by anion-exchange HPLC using a Partisil 10-SAX column and a discontinuous gradient of 1.25 M NaH2PO4 (pH 3.7) at a flow rate of 1 mL min−1 (Munnik, 2013). Fractions were collected every 30 s and measured for radioactivity by liquid-scintillation counting.

NORTHERN BLOT ANALYSIS

Total RNAs were isolated from seedlings ground in liquid nitrogen using the guanidinium thiocyanate-CsCl purification method (Sambrook et al., 1989). RNAs were separated by electrophoresis in a 1.2% agarose-formaldehyde gel. After transfer to nylon membrane, RNAs were fixed by UV cross-linking. Membranes

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Regulation of proline catabolism by Phosphatidylinositol 3-kinase

MATERIALS AND METHODS

PLANT MATERIAL

Arabidopsis (Arabidopsis thaliana) Heynh, ecotype Columbia Col-0 as wild-type (WT) and pi3k hemizygous mutant from GABI library (GK_418H02-018138) were used. In the hemizygous pi3k mutant (pi3k/pi3k), T-DNA insertion is located in the fifth exon of one allele of the gene (Lee et al., 2008b). WT seeds were sown on 0.5 × Murashige and Skoog (MS) solid medium (0.8% agar) in 14-cm-diameter Petri dishes as described previously (Parre et al., 2007). pi3k mutant seeds were sown on 0.5 × MS solid medium supplemented with 19.2 µM sulfadiazine (dissolved in DMSO) in order to select hemizygous plants versus WT homozygous plants. After 16 h at 4°C to raise dormancy, seeds were germinated and grown under continuous light with an intensity of 90 µmol m−2 s−1 for 12 days at 22°C.

STRESS AND PHARMACOLOGICAL TREATMENTS

Twelve-days-old seedlings were removed from 0.5 × MS agar plates and put onto liquid 0.5 × MS medium (control) supplemented with either 200 mM NaCl or 400 mM mannitol. After different incubation times, seedlings were collected and immediately frozen in liquid nitrogen and stored at −80°C prior analysis.

For pharmacological treatments, seedlings were pre-treated for 1 h in 0.5 × MS liquid medium with various concentrations of LY294002 dissolved in DMSO or with the same amount of DMSO as a control. Seedlings were thereafter transferred for 3 h or 24 h onto 0.5 × MS liquid medium alone (control), or supplemented with either 200 mM NaCl or 400 mM mannitol and with the same amount of DMSO or LY294002 as for the pre-treatment.

PROLINE DETERMINATION

Free proline contents were measured using L-proline as a standard according to Bates et al. (1973).
were hybridized at 65°C with either specific 3'UTR region of *AtP5CS1* or with full length of *AtProDH1* according to Church and Gilbert (1984). The fragments were labeled with 32P-dCTP using Ready-To-GoTM DNA labeling beads. Before hybridization, membranes were stained with methylene blue as a control for RNA loading and transfer. The hybridization signals were quantified using a PhosphorImager (Amersham Biosciences, USA).

**QUANTITATIVE RT-qPCR ANALYSIS**
Total RNAs were extracted following the protocol of the RNeasy Plant Mini Kit (Qiagen) from around 100 mg of powder obtained after grinding a pool of seedlings. After treatment with the RNase-free DNase (Fermentas), 1.5 μg of total RNA were reverse-transcribed by Revertaid™ Reverse Transcriptase (Fermentas) using 1 μM oligodT following the manufacturer instructions. The resulting first-strand cDNA was 20-fold diluted and used as the template for real-time quantitative PCR (RT-qPCR) amplification performed on a MasterCycler® ep realplex thermocycler (Eppendorf) with Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific) following the manufacturer instructions. Each reaction was performed with 5 μL diluted cDNA sample in a total reaction volume of 15 μL. The relative expression of *P5CS1* (At2g39800), *ProDH1* (At3g30775) and *PI3K* (At1g60490) genes were determined using specific primers (Supplementary Table 1). Expression levels of the different genes were standardized to *APT1* (At1g27450) used as a standard reference. The applied RT-qPCR program was 2 min at 95°C, 40 cycles with 15 s at 95°C, 30 s at 55°C and 30 s at 72°C followed by 15 s at 95°C, 15 s at 55°C, a gradual temperature rise of 20 min to 55°C at 95°C associated with a streaming of the plate, followed by 15 s at 95°C. The expression level of each gene was calculated using the following equation: 

\[ \text{fold change} = 2^{\text{Ct}_{\text{gene}} - \text{Ct}_{\text{ref}}} \times 100. \]

**GEL ELECTROPHORESIS, ELECTRO-BLOTTING AND IMMUNOLOGICAL DETECTION**
Proteins were extracted as described in Martínez-García et al. (1999) separated by SDS-PAGE (Laemmli, 1970) and transferred electrophoretically to a nitrocellulose membrane in a solution of 48 mM Tris, 39 mM glycine, 0.04% (w/v) SDS and 20% (v/v) ethanol at 50 mA for 1 h. For immunodetection, the nitrocellulose membrane was incubated in TBS with 0.05% (v/v) Tween 20 (TBS-T) and 5% non-fat dry milk for 1 h at 4°C and then in TBS-T with 0.1% (v/v) rabbit anti-serum for 15 h at room temperature. Antiserums were obtained by immunization of rabbits with either P5CS or ProDH recombinant proteins (Thiery et al., 2004). Blots were washed with TBS-T. Detection was performed with an ECL assay using horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). Equal protein loading and integrity of protein samples were verified by Ponceau S red staining of the blot membrane.

**METABOLITE PROFILING USING GC-MS AND METABOLICOMICS DATA PROCESSING**
Three independent samples of 12-days-old seedlings from each genotype treated during 24 h in different conditions were collected, and the equivalent of 50 mg of powder of each samples were used to perform the extraction and further metabolomics analysis. Extraction, derivatization, analysis, and data processing were performed according to Fiehn (2006). Metabolites were analyzed by GC-MS 3 h after derivatization. One microliter of the derivatized samples was injected in splitless mode on an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass spectrometer. The column was an Rtx-5SilMS from Restek (30 m with 10-m Integra-Guard column). The liner (Restek 20994) was changed before each series of analyses, and 10 cm of column was removed. The oven temperature ramp was 70°C for 7 min then 10°C/min to 325°C for 4 min (run length 36.5 min). The helium constant flow was 1.5231 mL/min. Temperatures were as follows: injector, 250°C; transfer line, 290°C; source: 250°C; and quadrupole, 150°C. Samples and blanks were randomized. Amino acid standards were injected at the beginning and end of the analysis to monitor the derivatization stability. An alkane mix (C10, C12, C15, C19, C22, C28, C32, and C36) was injected in the middle of the queue for external calibration. Five scans per second were acquired.

Metabolites were annotated, and their levels on a fresh weight basis were normalized with respect to the ribitol internal standard.

Raw Agilent data files were converted in NetCDF format and analyzed with AMDIS (http://chemdata.nist.gov/mass-spc/amdis/). A home retention index/mass spectra library built from the NIST, Golm, and Fiehn databases and standard compounds were used for metabolite identification. Peak areas were then determined using the Quanlynx software (Waters) after conversion of the NetCDF file to masslynx format. TMEV (http://www.tm4.org/mev.html) was used for all statistical analysis. Univariate analysis by permutation (One-Way and Two-Way ANOVA) was first used to select the significant metabolites. Multivariate analysis (hierarchical clustering and principal component analysis) was then performed on this subset.

**RESULTS**

**LY294002 AFFECTS PROLINE ACCUMULATION ONLY IN RESPONSE TO SALT TREATMENT**
To investigate whether PI3K is involved in the regulation of proline metabolism in response to ionic and/or hyperosmotic constraints, the effect of LY294002 on proline accumulation was assessed in 12-days-old Arabidopsis seedlings subjected to either 200 mM NaCl or 400 mM mannitol for 24 h. As shown in Figure 1A, typically a 5- to 6-fold accumulation of proline is observed in Arabidopsis seedlings treated with either NaCl or mannitol in comparison to the control seedlings. Interestingly, while LY294002 had no effect on the proline levels in control seedlings or seedlings stressed with mannitol (Figure 1), 40% less proline accumulated in the LY294002-treated seedlings upon salt stress. When increasing concentrations of LY294002 were applied, proline levels decreased in a dose-dependent manner in plants stressed with NaCl, with a maximum effect observed for 100 μM LY294002 (Supplementary Figure 1). In contrast, no effect on the proline levels of control or mannitol-stressed seedlings where found, whatever concentration of LY294002 (Figure 1 and Supplementary Figure 1). These results show that LY294002 negatively regulates...
proline accumulation in response to salt stress but not to mannitol.

**LY294002 REDUCES THE LEVEL OF PI3P**

In order to characterize the inhibitory effect of LY294002 on PI3K activity, in vivo PI3P levels were measured. For practical reasons, 6-days-old seedlings were used. These seedlings accumulated slightly less proline than the 12-days-old seedlings after NaCl treatment but the results were consistent, indicating that the younger seedlings perceived and responded well to salt stress (data not shown).

Seedlings were $^{32}$P$_3$-labeled overnight and the lipids extracted and separated by TLC (Supplementary Figure 2). Phosphoinositides (PI, PIP, and PIP$_2$) were quantified using PhosphoImaging (Figure 2A). PI, a structural phospholipid of membranes, represented 11–12% of the total $^{32}$P-labeled phospholipids, and its levels remained fairly constant throughout our experiments. PIP and PIP$_2$ are minor lipid constituents and accounted for 1–2% and 0.1–0.15% of the total phospholipids, respectively. PIP$_2$ progressively increased upon salt stress, reaching a 3-fold increase at 3 h compared to control seedlings, while no significant effects of salt stress were found for PIP levels. Interestingly, LY294002 treatment caused a slight but significant decrease in PIP$_2$ under control conditions as well as in response to salt stress.

In plants, the PIP pool is composed of 3 isomers, PI3P, PI4P, and PI5P (Munnik and Vermeer, 2010). PI4P is the most predominant PIP species (~80–90%), with PI3P and PI5P each accounting for ~5–10% of the PIP pool (Meijer et al., 2001). On TLC, the PIP isomers cannot be separated but by removing their fatty acids and analysing the resulting glycerophosphoinositolphosphates (GroPInsPs) by HPLC, it is relatively easy to distinguish and quantify the GroPIns3P from the GroPIns4P and GroPIns5P. The latter two are rather difficult to separate (Meijer et al., 2001).

So to determine the PI3P levels under our conditions, TLC-separated $^{32}$P-labeled PIP spots from 30 min treated seedlings were scraped off, deacylated and separated by anion-exchange HPLC. At control and salt conditions, PI3P was found to account for ~5% of the PIP pool. Addition of 100 µM LY294002, however, induced a 50% decrease of PI3P, whatever control or stress condition (Figure 2B). These results, and the inhibitory effect of LY294002 on proline accumulation in response to salt stress, are consistent with the involvement of PI3P as a lipid mediator on the regulation of proline metabolism.
LY294002 IMPACTS THE EXPRESSION OF GENES INVOLVED IN PROLINE METABOLISM

Proline accumulation is the consequence of a tight regulation of gene expression (Szabados and Savouré, 2010; Liang et al., 2013). We investigated transcript accumulation of two genes involved in proline metabolism, P5CS1 and ProDH1 that encode key enzymes regulating proline biosynthesis and catabolism, respectively. RT-qPCR analysis showed a 17-fold higher AtP5CS1 transcript accumulation in seedlings upon 3 h salt stress than in control ones (Supplementary Figure 3), which lower to 3-fold at 24 h salt stress. In addition, salt stress induced a slight accumulation of AtProDH1 transcript but only after 24 h (Supplementary Figure 3).

The role of PI3K on key genes and enzymes involved in proline metabolism was investigated using LY294002. Northern and western blot analysis revealed that LY294002 affected mRNA and protein accumulation in all tested conditions. After 3 h LY294002 treatment, a modest increase of P5CS1 mRNA was observed in control condition while ProDH1 transcript level was higher (Figure 3A). A dramatic effect of LY294002 on both P5CS1 and ProDH1 expression compared to non-treated seedlings was observed at 24 h salt stress. In salt stress seedlings treated with LY294002, P5CS1 steady state transcript level was lower than in non-treated ones while ProDH1 transcript level was higher (Figure 3A). In salt stress seedlings treated with LY294002, P5CS1 transcript accumulation decreased by 60% (Figure 3C) compared to non-treated seedlings. RT-qPCR analysis confirmed the higher ProDH1 transcript accumulation in presence of LY294002 whatever the growth conditions (Figure 3C). Using western blots, LY294002 triggered P5CS accumulation in control seedlings while P5CS level diminished in salt-treated plantlets. In contrast, LY294002 strongly enhanced ProDH accumulation in both control and salt-treated seedlings (Figure 3B).

The lower proline accumulation observed in response to salt stress with LY294002 is correlated with a down-regulation of P5CS1 and up-regulation of ProDH1 at both transcript and protein levels. As LY294002 reduced P3SP levels, our data suggest that PI3K is involved in the regulation of proline metabolism.

**ProDH1 EXPRESSION IS INDUCED IN pi3k MUTANT**

To further unravel the role of PI3K in the regulation of proline metabolism, we aimed for Arabidopsis pi3k KO mutants. Unfortunately, however, homozygous pi3k mutants are not viable (Lee et al., 2008b; Gao and Zhang, 2012). To partly resolve this issue, we selected sulfadiazine-resistant seedlings to get hemizygous mutants (Lee et al., 2008b; Gao and Zhang, 2012). To partly resolve this issue, we selected sulfadiazine-resistant seedlings to get hemizygous mutants (Lee et al., 2008b; Gao and Zhang, 2012). Segregation analysis indicated a 1:1 ratio in sulfadiazine resistant and sensitive seedlings, respectively (data not shown), due to the male gametophytic defect (Lee et al., 2008b). Therefore PI3K/pi3k hemizygous mutants were selected and further analyzed (Figure 4A). PCR-based genotyping of GABI_418H02 pi3k mutants confirmed that no homozygous mutants were obtained from this selection (data not shown).

Expression analysis by RT-qPCR revealed a 25% decrease of steady-state PI3K transcript level in pi3k hemizygous mutants compared to WT (Figure 4B). In this mutant, ProDH1 transcript level was almost 5-fold higher than in WT seedlings in normal growth condition. On the contrary, no difference in P5CS1 transcript level was observed between WT and pi3k hemizygous mutant.

When WT and pi3k hemizygous mutant were subjected to 200 mM NaCl for 24 h, they showed a higher proline accumulation of 18-fold and 11.5-fold, respectively (Figure 5A). pi3k hemizygous mutant showed a lower proline accumulation in response to NaCl. However the proline accumulation was not significantly different from WT seedlings, probably due to the remaining PI3K wild-type allele.

P5CS1 and ProDH1 transcript levels were investigated in pi3k hemizygous seedlings in response to 3 and 24 h of salt stress (Figure 5B). P5CS1 mRNA accumulation was similar in WT and pi3k hemizygous in response to salt stress. An equivalent increase of P5CS1 transcripts was observed at 3 h of stress and a decrease at 24 h of stress. Higher ProDH1 transcript levels than WT were
LY294002 is an effect of the inhibitor (Leprince et al. Regulation of proline catabolism by Phosphatidylinositol 3-kinase). Hierarchic clustering analysis indicated that DMSO did not have any significant effect on the relative metabolites contents, indicating that the difference in the metabolite patterns obtained with LY294002 or DMSO upon control and salt stress conditions. To investigate other changes induced by LY294002, we compared the metabolite profiles of 12-days old WT seedlings treated with LY294002 or DMSO. Results indicate that pi3k hemizygous seedlings are able to respond to salt stress, triggering P5CS1 gene expression and proline accumulation, although the transcript level of ProDH1 is gene increased. Altogether, our data indicate that the higher level of ProDH1 transcripts is correlated with higher ProDH amount in pi3k mutant. These data are consistent with those obtained with LY294002 treatment (Figure 3), where LY294002 induced higher ProDH1 transcripts and proteins. These results further strengthen the participation of a PI3K-mediated pathway regulating proline catabolism.

LY294002 AFFECTS SEEDLING METABOLOME

To investigate other changes induced by LY294002, we compared the metabolite profiles of 12-days old WT seedlings treated with LY294002 or DMSO upon control and salt stress conditions. Hierarchic clustering analysis indicated that DMSO did not have any significant effect on the relative metabolites contents, indicating that the difference in the metabolite patterns obtained with LY294002 is an effect of the inhibitor (Figure 6). Treatment for 24 h of salt stress significantly modified the amounts of several metabolites. Relative amounts of sucrose, ribose and maltose as well as proline, serine and raffinose increased in response to salt stress (Figures 6, 7B). On the contrary, the amounts of some other sugars, like galactose, mannose, trehalose and xylose (Figure 6) and glucose-6-phosphate (Glucose-6-P) and fructose-6-phosphate (Fructose-6-P) decreased in response to salt stress (Figure 7A). LY294002 reduced the level of proline content almost by half (Figure 7B), in accordance with our previous results (Figure 1 and Supplementary Figure 1). Interestingly, two other compatible osmolytes, serine and raffinose, exhibited an accumulation pattern similar to that of proline, i.e., higher accumulation in response to salt stress and lower when LY294002 is added (Figure 7B). Surprisingly, LY294002 addition had a strong impact on some amino-acid levels whatever the treatment. The
most dramatic effect being for tyrosine with an almost 100-fold increase in the presence of LY294002, in either control condition or NaCl stress (Figure 7C). Similarly, a 10–20-fold increase was observed for lysine, leucine, isoleucine and phenylalanine and a 4-fold increase for valine in response to LY294002.

Thus, the strong increase of those aliphatic and aromatic amino acids in presence of LY294002 suggests that PI3K, and/or its product PI3P, negatively regulates their metabolism through inhibition of their synthesis and/or promotion of their catabolism.

DISCUSSION

In this paper, we investigated the effect of the PI3K inhibitor, LY294002, on the response of Arabidopsis seedlings to salt stress. LY294002 was found to reduce PI3P levels by 50% and to dramatically decrease the accumulation of proline upon salt stress. The latter was a consequence of lower transcript- and protein levels for P5CS1 and higher transcript and protein levels for ProDH1. In the pi3k hemizygous mutant line, as also observed for WT seedlings treated with LY294002, an up-regulation of ProDH1 expression was found, suggesting that PI3K and its product PI3P are involved
in a pathway repressing ProDH1 expression. Metabolomic profiling of Arabidopsis seedlings in response to salt stress showed that LY294002 reduced the amount of raffinose, another compatible osmolyte, and strongly increased the amount of free aliphatic and aromatic amino acids.

**NaCl Stress Modified Phosphoinositide Composition**

Pi is not only a structural phospholipid of membranes, but also a precursor of several signaling phosphoinositides that are produced by distinct kinases and phosphatases, which add and remove phosphates at different positions of the inositol ring (Mueller-Roeber and Pical, 2002; Xue et al., 2009; Munnik and Vermeer, 2010; Munnik and Nielsen, 2011). Characterization of the phospholipid composition of overnight 32P-labeled Arabidopsis seedlings showed that stress mostly affected the PIP2 pool. This latter is predominantly composed of the PI(4,5)P2 isomer (Munnik, 2013). Some plant systems, in particular Chlamydomonas, have reported on increased PI(3,5)P2 levels (Meijer et al., 1999), but we did not observe this here for Arabidopsis seedlings. Increased PIP2 levels in response to salt and/or osmotic stress have been reported for several plant systems (Pical et al., 1999; DeWald et al., 2001; Zonia and Munnik, 2004; Darwish et al., 2009; Munnik and Zarza, 2013). Part of this PI(4,5)P2 response occurs at the plasma membrane (Van Leeuwen et al., 2007; König et al., 2008), where it is considered to be an important signaling molecule (Munnik and Nielsen, 2011), potentially through initiation of vesicle budding via its interaction with clathrin-adapter proteins. The subsequent formation of clathrin-coated vesicles during salt stress could be a mechanism for the cell to modify the plasma membrane according to water/ion movement (König et al., 2008). E.g., PI(4,5)P2 has been suggested to regulate stomatal aperture in response to water stress by regulating K+-efflux channel (Lee and Lee, 2008). Alternatively, PI(4,5)P2 can be hydrolysed by PLC to form inositol trisphosphate (IP3) and DAG, which can both be rapidly metabolized into other signaling molecules, e.g., IP6 and phosphatidic acid (Munnik and Vermeer, 2010). PLC has been implicated in salt stress signaling (Drobak and Watkins, 2000; Tasma et al., 2008; Xue et al., 2009; Munnik and Vermeer, 2010). In addition, Parre et al. (2007) have demonstrated that proline biosynthesis in response to salt stress is regulated by a Ca2+-signature depending on PLC activity.

LY294002 addition had a small effect on the PIP2 pool. Theoretically, as an ATP analog, LY294002 could inhibit other PLC substrates contributing to the reduced P5CS1 expression. The comparison between seedlings treated with LY and pi3k mutant showed differences in P5CS1 expression in contrast to ProDH expression. This may be due to the fact that LY294002 could
have additional effects like inhibiting other kinases. Another possibility is that adaptation to long-term decrease in PI3K activity could occur in the hemizygous pi3k mutant whereas the effect of LY294002 is more sudden and could change some of the plant stress responses.

Lee et al. (2008b) indicated that the pi3k mutant is strongly impaired in its pollen development, leading to the inability to obtain homozygous mutant. Our segregation analysis also showed a gametophytic defect in pi3k mutant, which explained why only pi3k hemizygous mutant could be obtained. pi3k mutant showed a reduced expression of the complete gene, in agreement with Leshem et al. (2007) and to the lethal phenotype of the reported antisense transformation (Welters et al., 1994). Consequently, genetic and biochemical analyses of PI3K are very difficult to assess. The development of promoter inducible lines may be useful to further investigate the role of PI3K in plant stress adaptation.

ProDH1 expression is under the control of bZIP10 and bZIP53 transcription factors (Satoh et al., 2004; Weltmeier et al., 2006; Dietrich et al., 2011; Veerabagu et al., 2014). Their expression and activity is regulated by various abiotic constraints, and also by the nutrient status of the plant (Weltmeier et al., 2009). In mammals, nutrient deficiency induces ProDH gene expression as a consequence of mTOR complex inactivation (Liao et al., 2008). VPS34 has been shown to participate in the regulation of mTOR upon nutrient stress (Backer, 2008). TOR is a protein kinase that possesses a catalytic domain with strong homology to the kinase domain of PI3K, and has also been shown to be sensitive to LY294002 (Brunn et al., 1996). In Arabidopsis, it is therefore possible that LY294002 inhibits TOR kinase too and as a result ProDH. Nevertheless, the fact that the ProDH1 increase is also observed in the pi3k hemizygous mutant background supports a direct involvement of the PI3K pathway in repressing ProDH1. TOR could even be a downstream component of PI3K and as such participate in the regulation of ProDH expression. This has already been observed in other eukaryotes (Liao et al., 2008).

**BESIDES PROLINE ACCUMULATION, LY294002 AFFECTS PLANT METABOLISM**

Salt stress affects several metabolites, among them sugars such as hexose-phosphates, disaccharides and raffinose family oligosaccharides (RFO). Sucrose, maltose and raffinose accumulated in response to salt stress, whereas Glu-6-P and Fru-6-P decreased. Variations of these sugar contents are well-conserved responses among various plant species upon salt stress (Kempa et al., 2008; Sanchez et al., 2008). These sugars are derived from photosynthesis activity. They have a key role in osmoprotection, in ROS scavenging and as a source of carbon storage (Keuen et al., 2013; Gupta and Huang, 2014). Sugar could also be considered as signaling molecules regulating gene expression and triggering specific responses to abiotic stress (Keuen et al., 2013). Raffinose is the only sugar whose accumulation was reduced by 50% in salt-stressed Arabidopsis seedlings treated with LY294002. Similar to proline, raffinose is also considered as a compatible solute for plant cells (Valluru and Van den Ende, 2011; Van den Ende, 2013). In order to determine whether the biosynthesis of raffinose upon salt stress is dependent of the same signaling pathway as proline, it will be of interest to investigate the regulation of raffinose metabolism in pi3k mutant background.

The accumulation of several amino acids other than proline was observed upon salt stress. Aliphatic (Val, Leu, Ileu) and aromatic (Phe, Tyr) amino acids increased in response to salt stress (our study; Kempa et al., 2008; Sanchez et al., 2008), but also after an extended period of darkness (Usadel et al., 2008). These authors suggested that these might be due to induction of protein catabolism and/or remobilization of nitrogen sources. Surprisingly, a dramatic increase of these amino acids was observed in seedlings treated with LY294002 grown in normal conditions. Further experiments will be required to determine the role of PI3K signaling in the regulation of amino acid metabolism.

In conclusion, our results strongly indicate that a signaling pathway involving PI3K and its product PI3P is involved in the repression of proline catabolism upon salt stress. Identifying specific PI3P targets will allow to decipher whether TOR is an intermediate signaling component in the regulation of ProDH in plants.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: [http://www.frontiersin.org/Journal/10.3389/fpls.2014.00772/abstract](http://www.frontiersin.org/Journal/10.3389/fpls.2014.00772/abstract)

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