The Yeast HAL2 Nucleotidase Is an in Vivo Target of Salt Toxicity*

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José Ramón Murguía, José María Bellés, and Ramón Serrano§
From the Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia–Consejo Superior de Investigaciones Científicas, Valencia, Spain

The yeast halotolerance gene HAL2 encodes a nucleotidase that dephosphorylates 3’-phosphoadenosine 5’-phosphate (PAP) and 3’-phosphoadenosine 5’-phosphosulfate (PAPS), intermediates of the sulfate assimilation pathway. This nucleotidase is inhibited by Na⁺ and Li⁺ but not by K⁺. Incubation of wild-type yeast cells with NaCl and LiCl, but not with KCl, increased intracellular PAP to millimolar concentrations. No depletion of the pool of adenine nucleotides (AMP, ADP, ATP) was observed. Other stresses such as heat shock or oxidative stress did not result in PAP accumulation. PAPS concentrations also increased during salt stress but remained lower than 0.5 μM. S-Adenosylmethionine concentrations decreased by 50%, reflecting inhibition of sulfate assimilation during salt stress. Salt-induced PAP accumulation was attenuated in a yeast strain overexpressing HAL2. This strain grew better than the wild type under salt stress. These results suggest that the cation sensitivity of the HAL2 nucleotidase is an important determinant of the inhibition of yeast growth by sodium and lithium salts. In addition to blocking sulfate assimilation by product inhibition of PAPS reductase, PAP accumulation may have other unidentified toxic effects.

The progressive salinization of irrigated land compromises the future of agriculture in arid regions, and it has turned the genetic improvement of salinity tolerance into an urgent biotechnological goal (1, 2). The identification of crucial metabolic reactions sensitive to salt may provide tools for the genetic engineering of salt tolerance (3, 4). High salt concentrations (>0.3–0.5 M NaCl) inhibit most enzymes because of the perturbation of the hydrophobic-electrostatic balance between the forces maintaining protein structure (5). It seems that many metabolic reactions and membrane functions would be strongly affected by these salt concentrations (5–7). In addition to these relatively nonspecific salt effects, some enzymes may be specially sensitive to inhibition by Na⁺ or Cl⁻ at much lower concentrations. The identification of these more specific targets is of crucial importance for the understanding of salt damage and for the manipulation of salt tolerance. Ribosomal protein synthesis has been considered a possible salinity target because of the salt sensitivity exhibited in vitro (5). In addition, we could expect sodium sensitivity in the case of potassium-activated enzymes, such as some reactions of sugar metabolism (5), and chloride sensitivity in the case of anion-utilizing enzymes (6, 7). No evidence exists about the physiological relevance of any of these potential targets of salt toxicity.

An approach to the identification of cellular processes most crucial to salt tolerance under in vivo conditions has been the cloning of yeast genes that by overexpression improve growth under salt stress. Three halotolerance genes have already been isolated. HAL1 (3) and HAL3 (8) modulate intracellular sodium and potassium concentrations and encode components of the regulatory machinery for ion homeostasis. HAL2, on the other hand, does not affect ion homeostasis but is required for methionine biosynthesis and is allelic to MET22 (4). This suggested that a particular enzyme of the methionine-biosynthetic pathway was salt sensitive. The prediction that methionine supplementation should therefore improve salt tolerance could be demonstrated (4).

HAL2 corresponds to a side reaction essential for sulfate assimilation. Inorganic sulfate is activated in two steps, consuming two ATP molecules, and is accumulated in the form of 3’-phosphoadenosine 5’-phosphosulfate (PAPS).1 When this activated sulfate is used by either reduction to sulfite or transfer to other molecules, a nucleotide is formed, 3’-phosphoadenosine 5’-phosphate (PAP), which must be hydrolyzed to AMP to recycle adenosine (9). The nucleotidase catalyzing this reaction is encoded by the yeast HAL2/MET22 gene, and it is very sensitive to inhibition by lithium (Ki = 0.1 mM) and sodium (Ki = 20 mM). Excess potassium (>0.1 M) counteracts the inhibition produced by these toxic cations (10).

In this work, we examine whether the HAL2 nucleotidase is an in vivo target of salt toxicity by measuring the accumulation of its substrates, PAP and PAPS, in salt-stressed cells. The results demonstrate a correlation between PAP accumulation and growth inhibition by salt, suggesting that the HAL2 nucleotidase is an important target of salt toxicity under physiological conditions.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions—Two Saccharomyces cerevisiae strains derived from RS-16 (MATa leu2-3, 112 ura3-251, 328, 372 (3)) were used. Wild-type strain RS-41 corresponded to RS-16 transformed with control plasmids Ycp50 (URA3) and pSB32 (LEU2) (11) to complement the uracil and leucine auxotrophies. The HAL2-overexpressing strain JRM8 or YEP-HAL2 (10) corresponded to strain RS-1051 (RS-16 with a hal2::URA3 disruption (4)) transformed with expression plasmid pJR5 (open reading frame of HAL2 in the LEU2 expression plasmid pRS-1024 (12)). Yeast cells were grown at 30 °C in minimal synthetic dextrose medium containing 2% glucose, 0.7% yeast

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The abbreviations used are: PAPS, 3’-phosphoadenosine 5’-phosphosulfate; PAP, 3’-phosphoadenosine 5’-phosphate; APS, adenosine 5’-phosphosulfate; AdoMet, S-adenosylmethionine; MES, 4-morpholineethanesulfonic acid; HPLC, high pressure liquid chromatography.
nitrogen base without amino acids (Difco), and 50 mM MES adjusted to pH 6.0 with Triz. LiCl, NaCl, or KCl was added as indicated. Salt tolerance in liquid medium was determined as described previously (3, 4).

Preparation of 35S-labeled Extracts and Two-Dimensional TLC Analysis—Yeast cells were grown in sulfate-free minimal medium (13) supplemented with 0.15 mM [35S]sulfate (675 Ci/mol) (Amersham). LiCl (0.1 mM) was added when cells reached an absorbance at 660 nm of 0.4–0.5 (exponential phase; measured with an Spectronic 20, Milton Roy Co., Rochester, NY), and the incubation was continued for 2 h. One ml of the labeled culture was centrifuged (30 s at 10,000 rpm), and the cell pellet was extracted with 200 μl of 1 mM formic acid during 30 min at 0°C and frozen overnight at −70°C. The next day samples were thawed, clarified by centrifugation, lyophilized, and resuspended in 5 μl of water. Four μl were applied as spots on polyethyleneimine cellulose plates (20 × 20 cm; Sigma) and analyzed by two-dimensional TLC as described (14). Standard compounds were co-chromatographed with the 35S-labeled extracts and located under UV light. The 35S-labeled compounds were visualized by autoradiography, quantitated by scintillation counting, and converted to intracellular concentrations. Intracellular water content was measured as described previously (15).

Preparation of Yeast Nucleotide Extracts and HPLC Analysis—Yeast cells were grown to an absorbance at 660 nm of 0.3–0.4 (exponential phase) and then treated with 0.1 mM LiCl, 1 mM NaCl, or 1 mM KCl. At the indicated times, 50-ml samples were harvested by filtration and immediately extracted with 1 ml of 2N perchloric acid at 0°C for 15 min. Extracts were clarified by centrifugation at 2000 rpm for 5 min, neutralized with 1 ml of 1.8 M KOH and 0.4 mM KHCO3, and centrifuged again as above. Supernatants were dried in a Speed-Vac concentrator (Savant), resuspended in 100 μl of water, filtered through Millipore HV filters (0.45 μm), and stored at −70°C. From 5 to 10 μl of each extract were analyzed by HPLC in a Waters 600E liquid chromatograph. Samples were injected onto a reversed phase C18 column (LiChrospher 100, 4 × 250 mm, 5-μm particle size, Merck), eluted, and detected as described (16). Nucleotide peaks were identified by co-injection with standards and, in the case of PAP, also by peak shift after treatment with purified HAL2 nucleotidase (10). Peak areas were quantified with a Waters 746 integrator and converted to millimolar concentrations by comparison with known amounts of PAP, AMP, ADP, and ATP standards. Intracellular water content was considered as described above. 3'-AMP was added routinely as an internal standard before extraction with perchloric acid. Recovery of this nucleotide was ≥90%.

Measurement of Intracellular Ion Concentration—After incubation in medium containing 0.1 mM LiCl as described above, samples of 10 ml were collected at the indicated times, processed, and analyzed by atomic absorption spectrophotometry as described (8).

RESULTS AND DISCUSSION

LiCl Stress Produces an Accumulation of PAP in Yeast Cells—Previous in vitro studies have shown that the HAL2 nucleotidase is inhibited by sodium and lithium ions (10). Therefore, if this enzyme is actually inhibited under in vivo conditions by these toxic cations, we should be able to detect an internal accumulation of its substrate PAP in cells experiencing sodium or lithium stress. To test this hypothesis, we performed HPLC analysis of nucleotide extracts from control (Fig. 1A) and LiCl-treated (Fig. 1B) wild-type yeast cells. Nucleotide peaks corresponding to AMP, ADP, and ATP were identified in the extracts. The most significant difference after lithium treatment was the appearance of a nucleotide peak that was absent in control extracts. This peak had the same retention time as the PAP standard, and it disappeared after incubation of the extracts with purified HAL2 nucleotidase (10), suggesting that it corresponded to intracellular PAP. Incubation with 1 mM NaCl also raised intracellular PAP but to lower levels than the LiCl treatment (Table I). This correlates with the much greater inhibition of the HAL2 nucleotidase by lithium than by sodium (10). Osmotic stress by KCl (Table I) or sorbitol (not shown) did not produce PAP accumulation. Additionally, neither oxidant stress (30 min with 0.5 mM H2O2) nor 0.5 mM H2S (30 min at 42°C) raised intracellular concentrations of PAP (not shown), suggesting that the accumulation of this nucleotide was specific for sodium and lithium stress.

Intracellular PAP could not be detected in control yeast cells, where its concentration must be lower than 10 μM (Table I). This is in agreement with the low Km for PAP (<20 μM) of the HAL2 nucleotidase (10). Other metabolites of the sulfate assimilation pathway such as APS and PAPS have steady-state concentrations that are under normal conditions <1 μM (14). Intracellular PAP concentrations increased after salt stress in a time-dependent manner (Fig. 2A). Substantial intracellular levels of this nucleotide (>0.5 mM) could be detected as soon as 15 min after LiCl treatment and reached a plateau at 2–3 mM in 2–4 h after salt stress. Intracellular lithium reached maximal concentrations of ~20 mM in 30–60 min of salt treatment (Fig. 2B). Intracellular potassium experienced a slight decrease (from 340 to 270 mM) during the first hour of LiCl treatment (Fig. 2B). As indicated in Table I, overexpression of HAL2 decreased PAP accumulation during salt stress. This suggests that PAP accumulation was actually due to inhibition of the HAL2 nucleotidase under in vivo conditions.

Sulfate Assimilation Is Inhibited in Lithium-stressed Yeast Cells—The increase in PAP levels produced by lithium could result in the product inhibition of the enzymatic reactions generating PAP from PAPS, such as PAPS reduction to sulfite during sulfate assimilation, therefore producing a blockade in the pathway. We have measured the intracellular concentrations of PAPS, which is produced immediately before the step where the HAL2 nucleotidase is operating, and AdoMet, one of the four metabolites of the activated methyl cycle (together with homocysteine, methionine, and S-adenosylhomocysteine) where sulfur is first assimilated (14, 17). As depicted in Fig. 3, lithium treatment raised intracellular PAPS concentrations (from 0.07 to 0.43 μM) while AdoMet concentrations experienced a 50% decrease. These results suggest an inhibition of the PAPS reductase step during lithium treatment because the concentration of one substrate of the reaction (PAPS) was increased while the concentration of one indirect product (AdoMet) was decreased. This effect can be explained by the known sensitivity of PAPS reductases from Escherichia coli.
Effects of salt stress on adenine nucleotide pool sizes and ratios in wild type (A) and HAL2 overexpressing (B) yeast strains

Exponentially grown cultures of each strain were incubated with the indicated salts for 2 h. After that, nucleotide extracts were prepared and analyzed by HPLC using the conditions described under "Materials and Methods." Data are given in mM concentrations and represent the mean ± S.D. of at least three independent experiments.

| Nucleotide | Control | 0.1 M LiCl | 1 M NaCl | 1 M KCl |
|------------|---------|------------|----------|---------|
| A. Wild-type strain |         |            |          |         |
| PAP        | N.D.    | 2.90 ± 0.50| 0.30 ± 0.05| N.D.    |
| ATP        | 3.50 ± 0.30| 2.20 ± 0.20| 2.20 ± 0.20| 3.00 ± 0.50|
| ADP        | 0.60 ± 0.10| 0.30 ± 0.10| 0.36 ± 0.08| 0.36 ± 0.06|
| AMP        | 0.10 ± 0.10| 0.40 ± 0.20| 0.36 ± 0.10| 0.25 ± 0.10|
| ΣAdN²     | 4.20 ± 0.30| 2.90 ± 0.50| 3.00 ± 0.38| 3.60 ± 0.60|
| AEC        | 0.90 ± 0.10| 0.78 ± 0.15| 0.80 ± 0.10| 0.88 ± 0.06|
| B. YEP-HAL2 strain |         |            |          |         |
| PAP        | N.D.    | 0.60 ± 0.30| 0.10 ± 0.02| N.D.    |
| ATP        | 3.60 ± 0.20| 2.30 ± 0.20| 2.20 ± 0.10| 3.27 ± 0.40|
| ADP        | 0.70 ± 0.10| 0.40 ± 0.07| 0.45 ± 0.06| 0.48 ± 0.10|
| AMP        | 0.20 ± 0.08| 0.30 ± 0.10| 0.25 ± 0.07| 0.25 ± 0.06|
| ΣAdN      | 4.50 ± 0.35| 0.30 ± 0.30| 2.90 ± 0.23| 4.00 ± 0.50|
| AEC        | 0.90 ± 0.10| 0.82 ± 0.07| 0.83 ± 0.06| 0.87 ± 0.05|

* N.D., not detected (<0.01).
* ΣAdN = |AMP| + |ATP| + |ADP|.
* AEC = ([ATP] + 1/2[ADP])/(|AMP| + |ATP| + |ADP|).

The Role of HAL2 Nucleotidase in Methionine Biosynthesis and Salt Tolerance—It must be concluded from these studies that the most significant change detected in yeast nucleotides and which correlates with growth inhibition under salt stress is PAP accumulation. This reflects an inhibition of the nucleotide pool sizes and ratios in wild type and in the HAL2-overproducing strain (Table I) and therefore does not correlate with either PAP accumulation or growth inhibition. NaCl produces a decrease in the energetic yield of yeast cultures because of an increased requirement of maintenance energy (21). This is probably concerned with maintaining an intracellular Na⁺ concentration lower than the extracellular concentration. A plausible mechanism is ATP consumption by the ENA1-encoded sodium (and lithium) efflux ATPase (22). In addition, we can expect sodium and lithium inhibition of some enzymes of energy metabolism activated by potassium, such as pyruvate kinase and phosphofructokinase (5).

(18) and S. cerevisiae (19) to inhibition by PAP. Although the inhibition is a mixed type, Vmax is the most affected parameter, with a KI for PAP of 25 μM (18). It can be calculated that under salt stress, where PAP concentrations reach values of 0.3 (NaCl) and 2.9 (LiCl) mM (Table I), the PAPS reductase must be strongly inhibited, >90% in the case of NaCl stress and >99% in the case of LiCl stress.

Intracellular PAPS concentrations during lithium treatment were 4 orders of magnitude lower than the measured concentration of PAP (Table I). A plausible explanation for this difference is based on the intrinsic chemical instability of PAPS, which is rapidly converted into PAP and sulfate in solution (20). It is likely that some of the PAP accumulated during salt stress derives not from the PAPS reductase reaction, inhibited under these conditions, but from spontaneous hydrolysis of the phosphosulfate bond of PAPS. Given the instability of PAPS and the very low concentrations of this metabolite measured under normal conditions, PAPS may exist in cells mostly bound to its producing and consuming enzymes, APS kinase and PAPS reductase, respectively (17). When it accumulates on inhibition of PAPS reductase, the free PAPS released from the enzymes could be spontaneously hydrolyzed into PAP and sulfate at high rates in the cytoplasmic environment.

Adenine Nucleotide Pools Are Not Depleted in Cells Subjected to Salt Stress—The in vitro inhibition of HAL2 nucleotidase by lithium and sodium implies that cells under salt stress are not able to convert PAP into AMP. This suggest that PAP could act as a sink for adenine nucleotides with dramatic consequences for cell growth. We have determined the intracellular contents of PAP, ATP, ADP, and AMP in control and salt-treated cells of wild-type yeast (Table IA) and HAL2-overexpressing yeast (YEP-HAL2) (Table IB), which exhibit different degrees of growth inhibition by salt (4). We have repeated these salt tolerance experiments because they are crucial for the interpretation of the nucleotide measurements discussed below and because the HAL2-overexpressing plasmid utilized in this work is different from the previous one. As described previously (4), NaCl and LiCl produced a much greater growth inhibition than KCl, which only poses osmotic stress. Overexpression of HAL2 improved growth with NaCl and LiCl but not with KCl (Fig. 4). NaCl and LiCl produced a reduction of ~30% in the total adenine nucleotide pool (AMP, ADP, and ATP), mainly due to a drop in ATP and ADP concentrations not compensated by a small increase in AMP. This depletion of adenine nucleotides is not prevented by overexpression of HAL2, which greatly decreases PAP accumulation and which improves growth under salt stress. Therefore, it is not relevant to the growth inhibition produced by sodium and lithium and cannot be ascribed to the inhibition of HAL2 nucleotidase and concomitant PAP accumulation. It probably corresponds to some other unidentified effect of sodium and lithium on yeast cells. This effect is largely specific for the toxic ions because KCl caused a much lower decrease of the total adenine nucleotide pool.

The decrease in the pool of adenine nucleotides produced by NaCl and LiCl, but not by KCl, correlates with a 10% decrease in adenylyl energy charge (from 0.9 to 0.8). Again, this change in energy status occurs similarly in the wild type and in the HAL2-overproducing strain (Table I) and therefore does not correlate with either PAP accumulation or growth inhibition. NaCl produces a decrease in the energetic yield of yeast cultures because of an increased requirement of maintenance energy (21). This is probably concerned with maintaining an intracellular Na⁺ concentration lower than the extracellular concentration. A plausible mechanism is ATP consumption by the ENA1-encoded sodium (and lithium) efflux ATPase (22).
explained by the above mechanism of HAL2 action: hal2
(mut22) mutants fail to grow with sulfur as sulfur source, while
PAPS reductase mutants (met16) can utilize sulfur (17). If the
HAL2 nucleotidase was required to prevent PAP accumulation
and if this accumulation had as its major effect the inhibition of
PAPS reductase, it cannot be understood why the requirement
for HAL2 cannot be bypassed by sulfite, the product of PAPS
reductase. We could explain the different growth responses of
hal2 mutants to methionine (growth) and to sulfite (no growth)
because methionine supplementation plays two roles: it pro-
vides a source of organic sulfur to bypass the inhibition of the
PAPS reductase reaction (something that sulfite could do); but
it also causes strong repression of all the genes involved in
sulfate assimilation (17, 23). Actually, a blockade of the pro-
duction of APS and PAPS by methionine supplementation has
been demonstrated (14). Therefore, methionine supplementation
would prevent PAP accumulation in the absence of HAL2
nucleotidase while sulfite supplementation could not. This
would lead to the proposal that PAP accumulation in the hal2
mutant has some toxic effect in yeast cells in addition to block-
ing sulfate assimilation.

In animal tissues, enzymatic sulfate transfers from PAPS to
proteins, polysaccharides, and small molecules constitute im-
portant metabolic reactions (20). PAPS sulfotransferases are
very sensitive to product inhibition by PAP, to the point that no
activity is detected in vitro in the absence of the nucleotidase
that converts PAP into AMP (24, 25). Sulfate transfer reactions
have also been described in plants (26) although not yet in
yeast. Another metabolic reaction sensitive to PAP accumula-
tion is the transfer of a phosphopantetheine group from CoA to
the acyl carrier protein. PAP is a product of the reaction, and it
strongly inhibits the enzyme holocarboxyl carrier protein synthase
(27). The accumulation of nonfunctional apoacyl carrier protein
will inhibit lipid metabolism, especially the acylation of sn-
glycerol-3-phosphate (28). A genetic approach is currently un-
der way to identify PAP-sensitive reactions relevant under in
vivo conditions.

HAL2 homologues have recently been identified in higher
plants such as the SAL1 gene of Arabidopsis thaliana (29) and
the RHL gene of rice (30). These genes are true functional
homologues because they complement the methionine auxotro-
phy of yeast hal2 mutants and they encode 3’/5’-bisphos-
phate nucleotidases with enzymatic properties that are similar
to the yeast enzyme. One important difference, however, is that

FIG. 2. PAP accumulation correlates with lithium uptake in
yeast cells exposed to LiCl. Exponentially growing cells (wild-type
strain RS-41) in minimal medium were supplemented at time zero with
0.1 M LiCl. Aliquots were taken at the indicated times, and internal
PAP and ion contents were determined as described under “Materials
and Methods.” A, time course of intracellular PAP accumulation. Data
represent the mean ± S.D. of at least three independent experiments.
B, internal K+ (○) and Li+ (■) concentrations at different times after
lithium addition. Essentially identical results were obtained in three
independent experiments.

FIG. 3. LiCl affects intracellular levels of AdoMet (SAM) and
PAPS in yeast cells. Yeast cells (wild-type strain RS-41) were grown
in 0.15 mM 35SO42− minimal medium. After 2 h of incubation with 0.1 M
LiCl, AdoMet (□) and PAPS (■) concentrations were measured as
described under “Materials and Methods.” Data represent the mean ±
S.D. of three independent experiments.

FIG. 4. Overexpression of HAL2 improves salt tolerance in
yeast cells. Saturated cultures of wild-type (WT) (□) and HAL2-over-
expressing (■) strains were diluted in fresh minimal medium with LiCl
(0.1 M), NaCl (1 M), or KCl (1 M) as indicated. Growth was recorded after
18 h. The results shown are the mean ± S.D. of three independent
experiments, each one done in duplicate.
the *Arabidopsis SAL1* nucleotidase has some activity (one-third than with PAP as substrate) with inositol 1,4-bisphosphate and inositol 1,3,4-triphosphate (29), typical substrates for inositol polyphosphate 1-phosphatases (31), regulatory enzymes that participate in the phosphoinositide-signaling pathway (32). 3(2’),5’-Bisphosphate nucleotidases and inositol polyphosphate 1-phosphatases are considered distinct enzymes, with code numbers EC 3.1.3.7 and EC 3.1.3.57, respectively (33). The enzymatic properties of the *Arabidopsis SAL1* phosphatase may suggest a revision of this distinction. Expression of *Arabidopsis SAL1* in yeast increased lithium and sodium efflux (29); therefore, it has been proposed that the product of the SAL1 gene has two functions; it participates both in the sulfur assimilation pathway and in the phosphoinositide-signaling pathway, changes in the latter probably affecting cation transport (29). Overexpression of HAL2 does not affect intracellular lithium and sodium concentrations, in agreement with the lack of activity of the HAL2 nucleotidase on inositol 1,4-bisphosphate (10). The activity of rice RHL nucleotidase on inositol 1,4-bisphosphate and its effects on salt tolerance and cation transport by yeast cells have not been investigated (30).

The proposal made for *E. coli* (34) and for *Chlorella* (35) that the physiological substrate for 3(2’),5’-bisphosphate nucleotidases is PAPS must be evaluated in light of our experimental results. The fact that the rice RHL nucleotidase exhibits slightly lower *Kₘ* for PAPS than for PAP (*Kₘ* values of 100 μM versus 240 μM, respectively) has been taken as evidence for this proposal (30). However, enzyme *Kₘ* values should be related to intracellular substrate concentrations. Our measurements indicate that PAP, and not PAPS, is the metabolite that accumulates in vivo when the nucleotidase is inhibited by salt stress. As discussed above, PAPS is such an unstable molecule that it probably never reaches concentrations higher than a few micromolar/ liter, while the more stable PAP may reach millimolar concentrations. Therefore, we postulate that PAP is the physiologically relevant substrate for the HAL2 nucleotidase. In addition, the proposal that PAPS is a highly toxic metabolite with a concentration that must be controlled by a phosphatase (30, 34) does not fit the evidence that a limited accumulation of PAPS during heat shock (up to 10–20 μM) seems beneficial for yeast thermotolerance (14).

A final point of discussion is the possibility that the accumulation of PAP in cells exposed to sodium and lithium may serve a signaling role during salt stress responses. Given the multitude of cellular signaling molecules derived from nucleotides (36), this possibility deserves investigation.

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