DNA Sequences from Arabidopsis, Which Encode Protein Kinases and Function as Upstream Regulators of Snf1 in Yeast*

Sandra Hey†, Hubert Mayerhofer†‡, Nigel G. Halford‡, and J. Richard Dickinson†‡

From the †Department of Crop Performance and Improvement, Rothamsted Research, Harpenden ALS 2JQ, United Kingdom and the ‡Cardiff School of Biosciences, Cardiff University, P. O. Box 915, Cardiff CF10 3TL, United Kingdom

Sucrose nonfermenting-1 (Snf1)-related protein kinase-1 (SnRK1) of plants is a global regulator of carbon metabolism through the modulation of enzyme activity and gene expression. It is structurally and functionally related to the yeast protein kinase, Snf1, and to mammalian AMP-activated protein kinase. Two DNA sequences from Arabidopsis thaliana, previously known only by their data base accession numbers of NM_125448.3 (protein ID NP_200863) and NM_114393.3 (protein ID NP_566876) each functionally complemented a Saccharomyces cerevisiae elm1 sak1 tos3 triple mutant. This indicates that the Arabidopsis proteins are able to substitute for one of the missing yeast upstream kinases, which are required for activity of Snf1. Both plant proteins were shown to phosphorylate a peptide with the amino acid sequence of the phosphorylation site in the T-loop of SnRK1 and by inference SnRK1 in Arabidopsis. The proteins encoded by NM_125448.3 and NM_114393.3 have been named AtSnAK1 and AtSnAK2 (Arabidopsis thaliana SnRK1-activating kinase), respectively. We believe this is the first time that upstream activators of SnRK1 have been described in any plant species.

Sucrose nonfermenting-1 (Snf1)3-related protein kinase-1 (SnRK1) of plants is a metabolic regulator that is activated in response to high cellular sucrose/low cellular glucose and regulates carbon metabolism through the modulation of enzyme activity and gene expression (1, 2). It is structurally and functionally related to the yeast protein kinase, Snf1, which gives the family its name, and to mammalian AMP-activated protein kinase (AMPK). SnRK1 phosphorylates and inactivates 3-hydroxy-3-methylglutaryl-coenzyme A reductase, sucrose phosphate synthase, nitrate reductase, trehalose-phosphate synthase and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (2–4). In addition, SnRK1 is required for redox modulation of ADP-glucose pyrophosphorylase activity in response to sucrose (5) and, like its fungal and animal counterparts, is involved in regulating the expression of genes involved in carbon metabolism (2). For example, overexpression of Snf1 in potato tubers causes an increase in starch content and a decrease in glucose levels resulting from a dramatic increase in the level of expression and activity of two key enzymes in the starch biosynthetic pathway, sucrose synthase and ADP-glucose pyrophosphorylase (6).

SnRK1 is regulated in part by phosphorylation on a threonine residue within the so-called “T-loop” (7) (Thr-173 in rye SnRK1 (8)), but the protein kinase responsible for this phosphorylation has hitherto proved elusive. The T-loop or “activation segment” is a region where many other protein kinases are activated by phosphorylation (9), including the mammalian homologue of SnRK1, AMPK, and the yeast homologue, Snf1 (10, 11). Furthermore, dephosphorylated, inactive Snf1 can be reactivated using the purified mammalian upstream kinase (12).

Attempts to identify and clone protein kinases that phosphorylate Snf1 have been given a new impetus by the identification of orthologous protein kinases in mammals (LKB1 and CaMKK) (4, 13–15) and the yeast Saccharomyces cerevisiae (Elm1, Sak1, and Tos3) (16, 17). Yeast offers some particular experimental advantages. For example, a yeast snf1 mutant is unable to derepress glucose-repressible activities and cannot grow on alternative carbon sources to glucose such as sucrose or nonfermentable carbon compounds such as glyceral or ethanol. Also, an elm1 sak1 tos3 triple mutant is unable to activate its Snf1 protein kinase; hence it too has a snf1− phenotype and behaves like a snf1 mutant. This provides a simple way to test DNA sequences which potentially encode orthologs of the yeast Elm1, Sak1 and Tos3 proteins, since the expression of a single ortholog in a yeast elm1 sak1 tos3 triple mutant will restore growth on sucrose and nonfermentable carbon sources. We have used this approach to confirm that two Arabidopsis (Arabidopsis thaliana) DNA sequences, hitherto known only by their data base accession numbers of NM_125448.3 (protein ID NP_200863) and NM_114393.3 (protein ID NP_566876) each functionally complement a yeast elm1 sak1 tos3 triple mutant. We further show that both plant proteins can phosphorylate a peptide with the amino acid sequence of the phosphorylation site in the T-loop of Snf1.
Data Base Searches—Searches of the Arabidopsis genome gene products were performed using the BLAST facility at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The amino acid sequences used for the initial searches were those given in data base entries NP_011336 (Tos3), AAA02892 (Elm1) and NP_011055 (Sak1).

Yeast Strains, Media, and Growth Conditions—S. cerevisiae wild type strain MSY182 (MATa ura3–52 leu2Δ0 his3Δ200 trplΔ63) and mutant strain MSY857 (MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ sak1::KANMX4 elm1::KANMX4 tos3::KANMX4) were kindly provided by Martin Schmidt (University of Pittsburgh, Pittsburgh, PA). Complex media comprised per liter yeast extract (10 g), bacteriological peptone (20 g), adenine (0.1 g), uracil (0.1 g), and either glucose (20 g) (in YPD) or glycerol (30 ml) in YPG. Synthetic complete media comprised per liter BactoYeast Nitrogen Base (BD Biosciences) (1.67 g), ammonium sulfate (5 g) and all appropriate supplements (20 mg of each) with either glucose (20 g) (“SC-glucose”) or glycerol (30 ml) and ethanol (20 ml) (“SC-glycerol-ethanol”). Agar (2% w/v) was used to solidify the media.

Plasmids, Transformation, and Plasmid Curing—Plasmid pVTL260, which has leu2d as the selectable marker and uses the yeast ADH1 promoter and terminator for expression in yeast, was obtained from EUROSCARF (Frankfurt am Main, Germany). The two Arabidopsis DNA sequences were generated by reverse transcription-PCR using primers designed to amplify between (and including) the initial methionine and the N terminus of the expressed proteins. The new plasmids were sought which could grow on synthetic complete medium with glycerol and ethanol as carbon source. Putative transformants were “cured” of plasmid by 3×-serial subculture (0.5 ml into 100 ml of liquid YPD medium at 30 °C for 24 h, where the plasmid is not required for growth) and finally plated onto solid YPD medium. Individual colonies were subsequently picked and patched onto a fresh YPD plate. After 2–3 days, the patches on the YPD plate were replica-plated to YPD, SC-glucose, and SC-glycerol-ethanol plates. Clones, which were no longer capable of growth on SC-glycerol-ethanol, were examined for the presence of the original plasmid and compared with the initial transformants and those cells that retained the ability to grow on SC-glycerol-ethanol.

Protein Kinase Assays—Yeast strains were grown in YP medium (1% yeast extract, 2% Bacto Peptone) including glucose at 2% (w/v) at 30 °C until the A600 reached ~2.0. The cells were collected by centrifugation at 2000 × g for 2 min, washed twice in “repressing medium” (fresh medium containing 2% (w/v) glucose, 0.05% (w/v) sucrose) or “derepressing medium” (fresh medium containing 0.05% (w/v) glucose, 0.05% (w/v) sucrose), as appropriate, then incubated for 30 min in the same medium. Harvesting was carried out as described (11) by rapid filtration onto Nylon-66 (0.45 μm pore size) filters. The cells were scraped off the membrane into 1.5-ml centrifuge tubes, snap-frozen in liquid N2, and stored at −80 °C.

Protein extracts were prepared according to Wilson et al., (11); 150 μl of extraction buffer (50 mM Na–HEPES, pH 7.0, 50 mM NaF, 50 mM NaCl, 1 mM EDTA, 10% (w/v) glycerol, 1 mM dithiothreitol, 1 mM benzamidine, 5 μg/ml soybean trypsin inhibitor, 1 mM phenylmethanesulfonyl fluoride) was added to the frozen cells, which were then thawed, suspended, and transferred to a 0.5-ml centrifuge tube containing 300 μl of acid-washed 0.4-μm glass beads on ice. The cells were disrupted by vortexing (5 × 30 s) and separated away from the glass beads by centrifugation at 2000 × g for 1 min. Cell debris was then removed by centrifugation at 14,000 × g for 1 min. An aliquot of the extract was removed for protein quantification using the method of Bradford (19) and the remainder snap-frozen in liquid N2 and stored at −80 °C.
The yeast extracts were assayed by measuring the rate of phosphorylation of the T-loop peptide (Ile-Met-Arg-Asp-Glu-His-Phe-Leu-Thr-Ser-Cys-Gly-Ser-Pro-Asn) using the assay described by Sugden et al. (20). Briefly, assays were carried out in a microtitre plate at 30 °C for 10 min, and each extract was assayed in triplicate with and without the T-loop peptide present. Each assay contained 5 μl of MgATP mix (1 mM ATP-NaOH, pH 7.0, 12.5 kBq [γ-33P]ATP, 25 mM MgCl₂), 7.5 μl of assay buffer (200 mM HEPES-NaOH, pH 7.5, 20 mM dithiothreitol, 5 mM Na₄PPi, 2.25 mM okadaic acid, 15 μl/ml protease inhibitor mixture (Sigma-Aldrich catalog number P9599)), 5 μl of either a 1 mM solution of the T-loop peptide or water, and 7.5 μl of crude protein extract (added to initiate the assay). An aliquot (15 μl) of the reaction was removed to Whatman P81 phosphocellulose paper (2 x 2 cm) and the reaction quenched by dropping the paper into a 1% phosphoric acid bath. The paper was washed five times in 1% phosphoric acid for 5 min each, then for 5 min in acetone, air-dried, and transferred to 3.5 ml of scintillation mixture for counting.

RESULTS

ORFs from Arabidopsis with High Sequence Similarity to Kinases in Yeast, Which Act Upstream of Snf1—Data base searches revealed that there are many ORFs in Arabidopsis that encode proteins with amino acid sequences similar to those of Elm1, Sak1, and Tos3 of S. cerevisiae but no “stand-out” obvious homologue (Table 1). Two sequences, NM_125448.3 (protein ID NP_200863) and NM_114393.3 (protein ID NP_566876), showed the highest similarity with both Tos3 and Sak1 and one of the highest similarities with Elm1. Nevertheless, a total of eight candidate sequences were amplified by reverse transcription-PCR from Arabidopsis seedling total RNA and cloned into the yeast expression vector for testing by functional complementation of a yeast elm1 sak1 tos3 “triple mutant.” Of these, six were cloned successfully (Table 1). Analysis of their nucleotide sequences confirmed identity with the sequences reported in the data base.

DNA Sequences from Arabidopsis, Which Functionally Complement a Yeast elm1 sak1 tos3 Triple Mutant—Wild type S. cerevisiae can utilize glucose and a wide range of nonfermentable carbon sources (e.g. sucrose, glycerol, and ethanol) (Fig. 1). The ability to use carbon sources other than glucose requires a fully functional Snf1. Since Snf1 function in yeast requires the upstream kinases Elm1 Sak1 and Tos3, an elm1 sak1 tos3 triple mutant cannot grow on glycerol (Fig. 1). However, transformation of strain MSY857 (elm1 sak1 tos3) with a plasmid expressing either NM_125448.3 or NM_114393.3 from Arabidopsis rendered it capable of growth on SC-glycerol-ethanol medium and other alternative carbon sources (Fig. 1). This implies that the Arabidopsis proteins encoded by NM_125448.3 and NM_114393.3 are able to substitute for one of the missing yeast upstream kinases. None of the other four Arabidopsis sequences tested showed this functional complementation.

Transformants expressing either NM_125448.3 or NM_114393.3 appear to be elongated (Fig. 2) showing that the plant kinases do not complement the elongation defect. A yeast elm1 sak1 tos3 mutant expressing a truncated version of Elm1 lacking the C-terminal domain has been shown to have a similar phenotype (21).

Plasmids of the expected size could be recovered from putative transformants that were capable of growth on glycerol (data not shown). Subjecting the putative transformants to a plasmid “curing” regime gave rise to a mixture of yeast clones: some were still able to grow on a nonfermentable carbon source, but some had now lost this ability (Fig. 3). It was not...
possible to demonstrate the presence of plasmid in those clones that had lost the ability to grow on a nonfermentable carbon source, whereas those that retained this ability still contained plasmid.

The Protein Kinases Encoded by NM_125448.3 and NM_114893.3 Phosphorylate a Peptide Containing the Activation Site within the T-loop of SnRK1—SnRK1 is regulated in part by phosphorylation on a threonine residue within the so-called T-loop (7), and the ability of the proteins encoded by NM_125448.3 and NM_114893.3 to phosphorylate this site was tested. Wild type yeast, mutant yeast strain MSY857 and MSY857 expressing either NM_125448.3 or NM_114893.3, using as substrate the T-loop peptide (Ile-Met-Arg-Asp-Glu-His-Phe-Leu-Lys-Thr-Ser-Cys-Gly-Ser-Pro-Asn), the amino acid sequence of which is identical to the activation site in the T-loop of SnRK1. The yeast strains were grown in YP medium containing 2% glucose and then transferred to medium containing 2% glucose, 0.05% sucrose for 30 min. Protein was extracted and assayed for the presence of an activity that could phosphorylate the T-loop peptide, the amino acid sequence of which Ile-Met-Arg-Asp-Glu-His-Phe-Leu-Lys-Thr-Ser-Cys-Gly-Ser-Pro-Asn is identical to the sequence within the “T-loop” activation site of SnRK1. There was some

FIGURE 2. The elongated morphology is retained. The elongated morphology is retained in colony 4 (MSY857 expressing Arabidopsis sequence NM_125448.3) (a), colony 7 (MSY857 expressing Arabidopsis sequence NM_114393.3) (b), and untransformed MSY857 (elm1 sak1 tos3) mutant (c). Colony 4 and colony 7 were grown on YP-glycerol; MSY857 cannot utilize glycerol, so it was grown on YPD.

FIGURE 3. Plasmid curing results in an inability to grow on a nonfermentable carbon source. Cells of colony 4 were subjected to a plasmid curing protocol. Individual clones recovered from a YPD plate were patched onto a fresh YPD plate. Next day they were replica-plated to YPD and YP-ethanol plates and incubated for 2 days at 30 °C. MSY857 (top, left-hand side) can grow on glucose but not on ethanol. Individual cells of colony 4, which had lost the plasmid, also lost the ability to grow on ethanol.

FIGURE 4. Protein kinase activity in various yeast strains. Protein kinase activity (pmol/min/mg) in wild type yeast, mutant yeast strain MSY857 and MSY857 expressing either NM_125448.3 or NM_114393.3, using as substrate the T-loop peptide (Ile-Met-Arg-Asp-Glu-His-Phe-Leu-Lys-Thr-Ser-Cys-Gly-Ser-Pro-Asn), the amino acid sequence of which is identical to the activation site in the T-loop of SnRK1. The yeast strains were grown in YP medium containing 2% glucose and then transferred to medium containing 2% glucose, 0.05% sucrose for 30 min. Bars represent 95% confidence level.
Figure 5. Alignment of the deduced amino acid sequences of the upstream protein kinases of Arabidopsis, yeast, and mouse. The sequences compared are AtSnAK1 and AtSnAK2 of Arabidopsis (*Arabidopsis thaliana*) with those of yeast (*Saccharomyces cerevisiae*) Elm1 (protein ID AAA02892), Sak1 (NP_011055) and Tos3 (NP_011336), and mouse (*Mus musculus*) LKB1 (BAA76749) and CaMKK1 (AAH17529).
Upstream Kinases in Arabidopsis

Animal LKB1-related

Animal CaMKK

Fungal CaMKK

Yeast Sakt-related

Plant SnAK

Protozoan CaMKK
Upstream Kinases in Arabidopsis

detectable “background” activity in the mutant strain, but much higher levels of activity in the wild type yeast (presumably attributable to the native yeast Sak1, Elm1, and Tos3 protein kinases) and the mutant strain expressing either NM_125448.3 or NM_114893.3 (Fig. 4).

Interestingly, this activity approximately doubled in the wild type yeast on transfer to derepressing conditions, while that of the mutant strain expressing the plant proteins declined (data not shown). Also unexpected was the fact that a sucrose spike (0.05% w/v) was necessary for activity to be detectable. Nevertheless, the result showed quite clearly that the protein kinases encoded by NM_125448.3 and NM_114893.3 could phosphorylate the T-loop peptide and by inference the activation site of SnRK1.

Upstream Activators of Snf1, AMPK, and SnRK1 Form a Family of Closely Related Protein Kinases—The proteins encoded by NM_125448.3 and NM_114393.3, which activate Snf1 in yeast and by inference SnRK1 of plants, were given the names AtSnAK1 and AtSnAK2 (Arabidopsis thaliana SnRK1-activating kinases). AtSnAK1 is a 407 amino acid protein with a molecular mass of 46 kDa, while AtSnAK2 comprises 396 amino acids and has a molecular mass of 45 kDa. Their amino acid sequences are aligned with those of Tos3, Sak1 and Elm1, as well as mammalian upstream activators of AMPK, LKB1, and CaMKII in Fig. 5. The protein kinase catalytic domains (Tyr-107 to Val-370 of AtSnAK1) align well, but there is little or no similarity between any of the proteins outside this domain. Indeed, the plant proteins have a relatively truncated C terminus, particularly when compared with Sak1, which comprises 1142 amino acids.

A protein-protein BLAST search of the National Center for Biotechnology Information “nr” data base was performed and the results are presented as a tree diagram (Fig. 6). The search identified SnAK-like sequences in alfalfa (Medicago sativa) and rice (Oryza sativa) (BE79890.1 and NP-001051055.1, respectively). All four plant SnAKs contain a Gly-Ser substitution in subdomain VII of the protein kinase catalytic domain (position 258 of AtSnAK1). This glycine residue forms part of the Asp-Phe-Gly triplet that is conserved in the vast majority of protein kinases. A BLAST search using the subdomain VII sequence from AtSnAK1/2 identified only two other protein kinases with the Asp-Phe-Ser triplet; these were two otherwise unrelated protein kinases from zebrafish (Danio rerio) (XP_684489.1 and XP_690087.1). There may be other protein kinases with the Asp-Phe-Ser triplet that were not identified in this search, but clearly the substitution is a rare one.

The BLAST search using the full-length AtSnAK1 sequence showed Sak1 and related protein kinases from other yeast species to be the most similar cluster to the SnAKs, followed by the animal CaMKK and LKB1-related protein kinases (Fig. 6). Tos3 and Elm1 are not similar specifically in response to sucrose. It is notable that activity of AtSnAK1 and AtSnAK2 could only be detected in the presence of a sucrose spike.

FIGURE 6. Tree diagram showing AtSnAK1, AtSnAK2, and related protein kinases. The diagram was created from a protein-protein BLAST search of the nr data base using the facility provided by the National Center for Biotechnology Information.

No other plant protein kinases appear in the figure. Indeed, the most similar group of plant protein kinases are the SnRKs, which, of course, form a separate cluster with the fungal and animal SNF1/AMPK-type protein kinases.

DISCUSSION

We have identified two sequences in Arabidopsis that will restore the ability of an elm1 sak1 tos3 triple mutant of the yeast S. cerevisiae to utilize nonfermentable carbon sources. The protein kinases encoded by these sequences, which have been given the names AtSnAK1 and AtSnAK2, will phosphorylate a peptide comprising the activation site in the T-loop of SnRK1. We believe this is the first time that such orthologous upstream activators have been described in Arabidopsis or any other plant species.

The members of the family of upstream activators of AMPK/Snf1/SnRK1 are clearly related and conserved, although it is notable that the degree of amino acid sequence similarity between the members of this family is considerably less than that between AMPK, Snf1, and SnRK1, which show ~50% amino acid sequence similarity with each other in the kinase catalytic domain and less but still significant similarity in the C-terminal regulatory domain (22).

The lack of similarity in the C-terminal domains may be indicative of the different proteins being involved in separate signaling pathways that converge on AMPK/Snf1/SnRK1. Detailed analysis of the C-terminal domains of the yeast proteins has revealed that these regions are essential for pathway specificity (21). Notably, deletion of the C-terminal domain of Elm1 has no effect on Snf1 signaling but prevents Elm1 from performing its normal function in the morphogenetic checkpoint signaling pathway. Cells lacking all three upstream kinases but transformed with a plasmid expressing an Elm1 lacking the C-terminal domain are able to activate Snf1 and utilize alternative carbon sources to glucose but are elongated, just like the cells described in the present study expressing AtSnAK1 or AtSnAK2. High levels of expression of Elm1 overcome the requirement for the C-terminal domain, and the cells have a normal morphology. The lack of similarity of the C-terminal domains of AtSnAK1 and AtSnAK2 with the much longer C-terminal domains of their yeast counterparts may also explain why the plant protein kinases do not respond to a shift from repressing to derepressing conditions.

The metabolite that initiates signaling through this pathway in mammalian systems is AMP, but the metabolites that are sensed in plant and fungal systems have never been identified satisfactorily (reviewed by Halford (2)). While there are many similarities between the animal, fungal, and plant systems there are also differences. For example, the plant SnRK1 pathway is the only one of the three to be activated specifically in response to sucrose. It is notable that activity of AtSnAK1 and AtSnAK2 could only be detected in the presence of a sucrose spike.
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REFERENCES

1. Halford, N. G., and Paul, M. J. (2003) Plant Biotech. J. 1, 381–398
2. Halford, N. G. (2006) Adv. Bot. Res. Incorp. Adv. Plant Pathol. 43, 93–142
3. Kulma, A., Villadsen, D., Campbell, D. G., Meek, S. E., Harthill, J. E., Nielsen, T. H., and MacKintosh, C. (2004) Plant J. 37, 654–667
4. Harthill, J. E., Meek, S. E., Morrice, N., Peggie, M. W., Borch, J., Wong, B. H., and MacKintosh, C. (2006) Plant J. 47, 211–223
5. Tiessen, A., Prescha, K., Branscheid, A., Palacios, N., McKibbin, R., Halford, N. G., and Geigenberger, P. (2003) Plant J. 35, 490–500
6. McKibbin, R. S., Muttucumaru, N., Paul, M. J., Powers, S. J., Burrell, M. M., Coates, S., Purcell, P. C., Tiessen, A., Geigenberger, P., and Halford, N. G. (2006) Plant Biotech. J. 4, 409–418
7. Sugden, C., Crawford, R. M., Halford, N. G., and Hardie, D. G. (1999) Plant J. 19, 433–439
8. Alderson, A., Sabelli, P. A., Dickinson, J. R., Cole, D., Richardson, M., Kreis, M., Shewry, P. R., and Halford, N. G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8602–8605
9. Johnson, L. N., Noble, M. E. M., and Owen, D. J. (1996) Cell 85, 149–158
10. Hawley, S. A., Davison, M., Woods, A., Davies, S. P., Beri, R. K., Carling, D., and Hardie, D. G. (1996) J. Biol. Chem. 271, 27879–27887
11. Wilson, W. A., Hawley, S. A., and Hardie, D. G. (1996) Curr. Biol. 6, 1426–1434
12. Mackintosh, R. W., Davies, S. P., Clarke, P. R., Weekes, J., Gillespie, I. G., Gibb, B. J., and Hardie, D. G. (1992) Eur. J. Biochem. 209, 923–931
13. Hurley, R. L., Anderson, K. A., Franzoni, J. M., Kemp, B. E., Means, A. R., and Witters, L. A. (2005) J. Biol. Chem. 280, 29060–29066
14. Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., Edelman, A. M., Frenguelli, B. G., and Hardie, D. G. (2005) Cell Metab. 2, 9–19
15. Woods, A., Dickerson, K., Heath, R., Hong, S. P., Momcilovic, M., Johnston, S. R., Carlson, M., and Carling, D. (2005) Cell Metab. 2, 21–33
16. Nath, N., McCartney, R. R., and Schmidt, M. C. (2003) Mol. Cell. Biol. 23, 3909–3917
17. Hong, S.-P., Leiper, F. C., Woods, A., Carling, D., and Carlson, M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 8839–8843
18. Ito, H., Funkuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163–168
19. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
20. Sugden, C., Donaghy, P., Halford, N. G., and Hardie, D. G. (1999) Plant Physiol. 120, 257–274
21. Rubenstein, E. M., McCartney, R. R., and Schmidt, M. C. (2006) Eukaryotic Cell 5, 620–627
22. Halford, N. G., and Hardie, D. G. (1998) Plant Mol. Biol. 37, 735–748