Identification of Nine Sucrose Nonfermenting 1-related Protein Kinases 2 Activated by Hyperosmotic and Saline Stresses in Arabidopsis thaliana*

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Several calcium-independent protein kinases were activated by hyperosmotic and saline stresses in Arabidopsis cell suspension. Similar activation profiles were also observed in seedlings exposed to hyperosmotic stress. One of them was identified to AtMPK6 (Droillard, M. J., Boudsocq, M., Barbier-Brygoo, H., and Laurieère, C. (2002) FEBS Lett. 527, 43–50) but the others remained to be identified. They were assumed to belong to the SNF1 (sucrose nonfermenting 1)-related protein kinase 2 (SnRK2) family, which constitutes a plant-specific kinase group. The 10 Arabidopsis SnRK2 were expressed both in cells and seedlings, making the whole SnRK2 family a suitable candidate. Using a family-specific antibody raised against the 10 SnRK2, we demonstrated that these non-MAPK protein kinases activated by hyperosmolarity in cell suspension were SnRK2 proteins. Then, the molecular identification of the involved SnRK2 was investigated by transient expression assays. Nine of the 10 SnRK2 were activated by hyperosmolarity induced by mannitol, as well as NaCl, indicating an important role of the SnRK2 family in osmotic signaling. In contrast, none of the SnRK2 were activated by cold treatment, whereas ascorbic acid only activated five of the nine SnRK2. The probable involvement of the different Arabidopsis SnRK2 in several abiotic transduction pathways is discussed.

Environmental stresses such as drought, cold, and salinity impose osmotic stress on plants, leading to imbalance in ionic homeostasis, oxidative damages, and growth inhibition. Understanding how plants respond to these stresses is critical to improve plant resistance. Reversible protein phosphorylation is one of the major mechanisms for mediating intracellular responses, including responses to osmotic changes. Indeed, several protein kinases have been shown to be activated by hyperosmotic stresses in different plant species. Because of the well-known osmosensing pathway in yeast involving a mitogen-activated protein kinase (MAPK),1 much interest was focused on the MAPK family in plants. In Arabidopsis, AtMPK6 and AtMPK4 were shown to be activated by hyperosmolarity, salt, cold, or drought (1, 2), whereas the tobacco SIPK was activated by hyperosmotic or salt stresses (3–5). In alfalfa, SAMK was reported to be activated by cold and drought but not NaCl (6), whereas SIMK was activated by sorbitol, KCl, and NaCl (7).

In mammals, MAPK cascades are composed of MAPK, MAPKK, and MAPKKK, each component being activated by phosphorylation by the upstream kinase. The involvement of MAPKK and MAPKKK in plant osmotic response was suggested by molecular and biochemical studies. The Arabidopsis MAPKKK AtMEKK1 was transcriptionally induced by salt stress and the protein was able to complement a yeast mutant affected in osmotic signaling (8). Moreover, Mizoguchi et al. (9) described using two-hybrid and yeast complementation a possible MAPK cascade composed of the osmotically activated AtMPK4, AtMEK1 (MAPKK), and AtMEKK1. More recently, the activation of AtMPK4 by AtMEK1 (10) and the interaction between SIPKK and SIPK (11) were demonstrated in vitro independently of osmotic signaling. Interestingly, Kiegerl et al. (12) demonstrated that SIMKK activated SIMK in vivo and enhanced the SIMK activation by NaCl.

Other kinase families have been shown to play a role in osmotic signaling. Among them, SOS2 (salt overly sensitive 2), which belongs to the sucrose nonfermenting 1-related protein kinase 3 (SnRK3) family, was transcriptionally induced by salt stress (13). sos2 mutant displayed hypersensitivity to Na+ ions but not to mannitol (14), suggesting a role of SOS2 in ion homeostasis. Using in vitro and yeast experiments, progress has been made in understanding the SOS2 pathway. Intramolecular interaction maintains SOS2 in an inactive form by autoinhibition (15), which is relieved by interaction with the calcium-binding protein SOS3 (16, 17). Then SOS3 targets SOS2 to the plasma membrane where the SOS2-SOS3 complex activates the SOS1 Na+ /H+ antiporter via phosphorylation (18, 19). Moreover, the complex also induces the transcriptional up-regulation of SOS1 by salt stress (20).

Calcium signals elicited by hyperosmotic stresses (21) can also be sensed by calcium-dependent protein kinases (CDPK), although very few data are available on the role of CDPK in osmotic signaling. Transcriptional induction of several CDPK was reported in response to salt, cold, or drought in different plant species (22–24). Moreover, overexpression of OsCDPK7 conferred cold, salt, and drought tolerance on rice plants (24). Interestingly, the activation of a rice CDPK in response to cold treatment was reported, but it occurred only 12 to 18 h after treatment, indicating that the kinase does not participate in the early response to stress but rather in the adaptive process (25).

Transcript accumulation in response to NaCl or PEG treatments was also reported for several SHAGGY/GSK3-like kinases (AtSK) (26, 27). On the other hand, AtSK22 complemented a yeast salt stress-sensitive mutant (26) and its overexpression enhanced salt and drought tolerance in Arabidopsis (28).

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‡ The abbreviations used are: MAPK, mitogen-activated protein kinase; SnRK2, sucrose nonfermenting 1-related protein kinase 2; SnRK3, sucrose nonfermenting 1-related protein kinase 3; CDPK, calcium-dependent protein kinase; ABA, abscisic acid; MES, 4-morpholine-ethanesulfonic acid; HA, hagemaglutinin; RT, reverse transcription; DTT, dithiothreitol; MBP, myelin basic protein.
Enzymatic restriction sites (KpnI, PstI, and SphI) are underlined. Oligonucleotides used for SnRK2 cloning into pQE30 and RT-PCR instructions (Invitrogen) using pGreen-HiA-GW (see manufacturer), the positive clones were verified by DNA sequencing. LR clonase recombination reactions to transfer DNA fragments from entry clones to destination vectors were carried out according to the manufacturer’s instructions (Invitrogen) using pGreen-HIA-GW (see below) as destination vector. The product of recombination reactions (LR reactions) was used to transform E. coli DH5α, and the positive clones were verified by DNA sequencing.

**Construction of Destination Vector** pGreen-HIA-GW—The blunt-ended Gateway conversation cassette C (Invitrogen) was inserted in the BamHI site of the intron-tagged HA-epitope cassette of p<i>PLY</i> vector (34) after filling in the site with Klenow polymerase I. The resulting cassette was inserted in the KpnI site of pGreen0129 (www. pgreen.ac.uk). This construction allows the expression of HA-tagged proteins under the control of a 35 S promoter carrying a duplicated enhancer domain.

**RT-PCR Analysis**—Total RNA was isolated from cell suspension 5 days after subculturing and from 6-day-old seedlings using Nucleospin RNA Plant kit (Macherey-Nagel). The cDNA was synthesized from 4 μg of total RNA and oligo(dT) using the Superscript First-Strand Synthesis System (Invitrogen). PCR was run for 30–40 cycles using the primers presented in Table I. Amplified transcripts were separated on 0.8% (w/v) agarose gel and detected by ethidium bromide staining. The gene specificity of the primers was confirmed by subsequent cloning and sequencing of the amplified products (see section “Expression and Purification of His-tagged SnRK2”). As an internal standard, the level of ACTN2 and ACTIN9 transcripts was monitored with the following primers: 5'-GAATGATCTGAGAAGTGGGC-3' (forward) and 5'-GCTGGAATGTGCTGAAGGAG-3' (reverse), because their combined expression profile was shown to be constitutive (35).

**Cell Suspension Treatments**—Osmolarity was monitored using a freezing-point osmometer (Roehling, Berlin, Germany). Cells were equilibrated for 4 h in their culture medium containing 10 mM MES-Tris, pH 6.2, and adjusted to 200 mM sucrose. After equilibration, extracellular medium was replaced by either the same volume of isoosmotic medium, 200 mM Osm (10 mM MES-Tris, pH 6.2, 1 mM CaSO<sub>4</sub>, 190 mM sucrose), or hyperosmotic medium, 500 or 1000 mM Osm (10 mM MES-Tris, pH 6.2, 1 mM CaSO<sub>4</sub>, 500 or 1000 mM sucrose). In the indicated cases, sucrose was replaced by 1000 mM mannitol or 650 mM sucrose to simulate hyperosmolarity. To stop treatments, suspension was filtered, frozen in liquid nitrogen, and stored at −80 °C until use.

**Preparation of Protoplasts**—Cell cultures were used 3 days after subculturing at 33% (w/v) for isolation of protoplasts. Cells were collected by centrifugation (40 × g for 5 min) and resuspended in 25 ml of enzymatic solution (1% (w/v) cellulase RS (Yakult, Tokyo, Japan), 0.2% (w/v) macerozyme R10 (Yakult) in modified JPL medium (JPL-A), which does not contain sucrose but 30.5 g/liter glucose and 30.5 g/liter mannitol). Final volume was adjusted to 75 ml with JPL-A and cells were incubated for 3 h 30 min on a rotary shaker in constant light. Protoplasts were collected by centrifugation (150 × g for 5 min), washed with JPL-A, and resuspended in JPL medium containing 0.28 mM sucrose. After centrifugation (150 × g for 5 min), floating protoplasts were harvested.

**Protoplast Transient Expression Assay**—Typically, 1.5 × 10<sup>6</sup> protoplasts in 120 μl were mixed with 20–40 μg of plasmid DNA and 380 μl of PEG solution (25% (w/v) PEG 6000, 450 mM mannitol, 100 mM Ca(NO<sub>3</sub>)<sub>2</sub>). After an incubation at 22 °C in the dark for 15 min, the transfection mixture was washed with 275 mM Ca(NO<sub>3</sub>)<sub>2</sub> and centrifuged (150 × g for 5 min). Protoplasts were resuspended in 50 μl of JPL-A and incubated in the dark at 22 °C for 15 h before treatments.

**Protoplast Treatments**—For osmotic stresses, protoplasts were centrifuged (150 × g for 5 min) and resuspended in the same volume of isoosmotic medium (JPL-A, 400 Osm) or hyperosmotic medium containing mannitol (JPL-A supplemented with 680 mM mannitol, 1000 mM NaCl and 350 mM mannitol) or 4°C for 10 min. For cold treatment, protoplasts were incubated for 10 min at 4 °C or room temperature for the control. For ABA treatment, protoplasts were centrifuged (150 × g for 5 min) and then the pellet was frozen in liquid nitrogen before storage at −80 °C until used.

**Preparation of Protein Extracts**—Cells or seedlings were ground in liquid nitrogen and homogenized at 4 °C in extraction buffer E1B (100 mM HEPEs, pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM orthovanadate, 10 mM NaF, 20 mM β-glycerophosphate, 5 mM DTT, 1 mM phenylmethyl-

**TABLE I**

| Gene     | Sequence of oligonucleotides forward (F) and reverse (R) |
|----------|--------------------------------------------------------|
| SnRK2-1  | 5'-TTGGATACGCTAAGGAACTGTACGTT-3'                      |
| SnRK2-2  | 5'-ATTGATACGCTAAAGGACTGTACGTT-3'                      |
| SnRK2-3  | 5'-TCTGGATACGCTAAGGAACTGTACGTT-3'                      |
| SnRK2-4  | 5'-TTGATACGCTAAGGAACTGTACGTT-3'                       |
| SnRK2-5  | 5'-TTGATACGCTAAGGAACTGTACGTT-3'                       |
| SnRK2-6  | 5'-TTGTACGCTAAGGAACTGTACGTT-3'                        |
| SnRK2-7  | 5'-TTGATACGCTAAGGAACTGTACGTT-3'                        |
| SnRK2-8  | 5'-TTGATACGCTAAGGAACTGTACGTT-3'                        |
| SnRK2-9  | 5'-TTGATACGCTAAGGAACTGTACGTT-3'                        |
| SnRK2-10 | 5'-TTGATACGCTAAGGAACTGTACGTT-3'                        |

Only few data are available on another group of the SNF1-related protein kinases (SnRK2), which appears unique to plants (29). A tobacco homolog of the Arabidopsis ASK1/SnRK2-4 was shown to be activated by hyperosmotic stress in cell suspension (4), whereas dehydration activated SnRK2-E/SnRK2-6 (30). In soybean, SPK3 and SPK4 were transcriptionally up-regulated by drought and saline stress (31), whereas only SPK1 and SPK2 were activated by salt stress in yeast (32).

We have previously reported the activation of several protein kinases that did not require calcium for their activity, in response to hyperosmotic stresses in Arabidopsis cell suspension (1). Among them, one was identified to the MAPK AtMPK6 but the others remained unknown. In this work, we demonstrated that these unidentified kinases were also activated in Arabidopsis seedlings and belonged to the SnRK2 family. Molecular identification revealed that nine of the 10 SnRK2 were activated by hyperosmolarity. Then the possible involvement of SnRK2 in other signal transduction pathways such as saline stress, cold, and abscisic acid (ABA) was also tested.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—Arabidopsis thaliana cell suspension (Columbia ecotype) was cultured in JPL medium as previously described (1) at 23 °C in constant light. Cells were used after 5 days subculturing with 100 mg of fresh weight/ml cell density.

A. thaliana (Columbia ecotype) seeds were sterilized and sown on a medium containing 5 mM KNO<sub>3</sub>, 2.5 mM KH<sub>2</sub>P<sub>4</sub>, 0.2 mM MgSO<sub>4</sub>, 1 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 1 mM MES, 5 μM Fe-EDTA, Murashige and Skoog microelements (33), 10 μl/liter sucrose, and 7 g/liter agar. The resulting cassette was inserted in the KpnI site of the intron-tagged HA-epitope cassette of p<i>PLY</i> vector (34) after filling in the site with Klenow polymerase I. The blunt-X fragment of recombination reactions (BP reactions) was used to transform E. coli DH5α.
sulfonfyl fluoride, 5 μM/ml leupeptin, 5 μg/ml antipain). After centrifugation at 17,600 × g at 4 °C for 15 min, the supernatant was precipitated in 10% (v/v) trichloroacetic acid solution containing 10 mM NaF, washed twice with 50% (v/v) cold acetone, and resuspended in SDS-PAGE sample buffer. Protein concentration was determined by the Bradford method (36). For immunoprecipitation, the 17,600 × g supernatant was observed as above in extraction buffer EB2 (EB1 modified in the concentration of three protectants: DTT (10 mM), orthovanadate (10 mM), and β-glycero-phosphate (60 mM)).

For protein extraction from protoplasts, extraction buffer EB3 corresponds to EB2 supplemented with 1% (v/v) Triton X-100 and 75 mM NaCl. The protoplast pellet was melted on ice with 100 μl of EB3 and vortexed. Protein extract was recovered after centrifugation at 17,600 × g at 4 °C, frozen in liquid nitrogen, and stored at −80 °C until use.

In-Gel Kinase Assay—Protein extract (20 μg) was separated on 10% SDS-polyacrylamide gels embedded with 0.2 mg/ml myelin basic protein (MBP) or 0.5 μg/ml histone as substrates for the kinases. The gels were treated for protein renaturation as described by Zhang et al. (37). For the activity, the gels were preincubated for 30 min at room temperature in kinase activity buffer (40 mM HEPES, pH 7.5, 2 mM DTT, 20 mM MgCl₂, 1 mM EGTA, 0.1 mM orthovanadate). Phosphorylation was performed for 1 h in 8 ml of the same buffer supplemented with 25 μM cold ATP and 2.9 MMBq of [γ-32P]ATP per gel. Then the gels were washed extensively in 5% (v/v) trichloroacetic acid and 1% (w/v) (w/v) diiodoacetamide pyrophosphate solution. The protein kinase activity was detected on the dried gels by the Storm imaging system (Amersham Biosciences).

Immunoprecipitation—Immunoprecipitation assays were carried out either with the polyclonal anti-HA antibody (Sigma) or with a polyclonal plant kinase antibody. It was raised against at least 5 amino acids of the catalytic domain of SnRK2 kinases (GYSKSSLLHSRPKST, Eurogentec). Protein extract (200 μg from cells or 100 μg from protoplasts) was incubated with either 35 μg of anti-SnRK2 or 1.6 μg of anti-HA (Sigma) antibodies in immunoprecipitation buffer (20 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 0.1 mM orthovanadate, 10 mM NaF, 60 μg β-glycero-phosphatase, 5 mM DTT, 1 mM phenethylsulfonfyl fluoride, 5 μg/ml leupeptin, 5 μg/ml antipain, 5 μg/ml leupeptin, 5 μg/ml antipain, 150 μg NaCl, 0.5% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40) for 3 h. Then 30 μl of 50% protein A-Sepharose CL-4B (Sigma) was added and incubation was continued for another 1 h. The immunoprecipitate was washed 4 times in immunoprecipitation buffer and twice in kinase buffer (20 mM Tris-HCl, pH 7.5, 12 mM MgCl₂, 2 mM EGTA, 2 mM DTT, 0.1 mM orthovanadate) and resuspended in SDS-PAGE sample buffer. Protein kinase activity of precipitated proteins was analyzed by in-gel kinase assay as previously described.

Results

Several Non-MAPK Protein Kinases Are Activated by Hyperosmotic and Saline Stresses in Arabidopsis Cells and Plants—To investigate the activation of Ca²⁺-independent protein kinases in response to hyperosmotic stresses, in-gel kinase assays were performed in the absence of calcium using MBP or histone as substrates. Moderate (500 mOsm) or high (1000 mOsm) hyperosmotic stresses were studied in comparison to an isoosmotic control condition as previously described (1). When Arabidopsis cell suspension was submitted to hyperosmolarity, the activation of several Ca²⁺-independent kinases was observed using MBP as a substrate: two thin activity bands with apparent molecular masses of 42 and 35 kDa and a thick one around 37–38 kDa (Fig. 1A). The activations were greater when the stress strength was increased. When the same extracts were analyzed using histone as a substrate, a similar activation profile was visualized, with two distinct bands with apparent molecular masses of 38 and 37 kDa in addition to the 42- and 35-kDa bands. These four protein kinases activated by hyperosmolarity can phosphorylate both histone and MBP, indicating that they do not belong to the MAPK family. By contrast, the 44-kDa kinase active on MBP but almost not on histone has already been identified to the MAPK AtMPK6 (1).

The effect of two other osmolytes, mannitol and NaCl, was analyzed in conditions leading to the same osmolarity (Fig. 1B). When cells were treated with 1 M sucrose, 1 M mannitol, or 650 mM NaCl, similar activation profiles were observed, indicating that these kinase activations represent a general response to osmotic stress.

| Gene | Sequence of oligonucleotides (forward) (F) and reverse (R) |
|------|----------------------------------------------------------|
| SnRK2-1 | F: 5′ – GGGGAACTTTTGTACAAAAAAGCAGGCTTCACCATGAGTACGCTT–3′ |
| SnRK2-2 | F: 5′ – GGGGACCACTTTTGTACAAGAAAGCTGGGGGACCACTTTGACAATGACGCTT–3′ |
| SnRK2-3 | F: 5′ – GGGGACCACTTTTGTACAAGAAAGCTGGGGGACCACTTTGACAATGACGCTT–3′ |
| SnRK2-4 | F: 5′ – GGGGACCACTTTTGTACAAGAAAGCTGGGGGACCACTTTGACAATGACGCTT–3′ |
| SnRK2-5 | F: 5′ – GGGGACCACTTTTGTACAAGAAAGCTGGGGGACCACTTTGACAATGACGCTT–3′ |
| SnRK2-6 | F: 5′ – GGGGACCACTTTTGTACAAGAAAGCTGGGGGACCACTTTGACAATGACGCTT–3′ |
| SnRK2-7 | F: 5′ – GGGGACCACTTTTGTACAAGAAAGCTGGGGGACCACTTTGACAATGACGCTT–3′ |
| SnRK2-8 | F: 5′ – GGGGACCACTTTTGTACAAGAAAGCTGGGGGACCACTTTGACAATGACGCTT–3′ |
| SnRK2-9 | F: 5′ – GGGGACCACTTTTGTACAAGAAAGCTGGGGGACCACTTTGACAATGACGCTT–3′ |
| SnRK2-10 | F: 5′ – GGGGACCACTTTTGTACAAGAAAGCTGGGGGACCACTTTGACAATGACGCTT–3′ |
Osmotic stress was induced by 1 M sucrose (a moderate hyperosmotic condition on cell suspension (500 mM isoosmotic medium), 1 M mannitol (isoosmotic medium), or 650 mM NaCl (high hyperosmotic medium)). Coherently, the 44 kDa displayed a strong activation in plantlets exposed to high stress, whereas the activity of this kinase decreased in cell suspension when the stress strength was decreased. As in cells, the 44-kDa kinase corresponds to AtMPK6 (1) is not immunoprecipitated by anti-SnRK2 antibody that succeeded in recognizing the 10 SnRK2 proteins (from 38 to 43 kDa). It should be noted that some faint additional bands can also be visualized that probably correspond to unspecific background and/or degradation products. To further characterize the antibody, its ability to recognize the 10 SnRK2 proteins was analyzed by immunoblotting on recombinant proteins. Each SnRK2 kinase was produced in bacteria with an His-tag and purified on nickel column. The recombinant proteins were immunoblotted with the anti-SnRK2 antibody that succeeded in recognizing the 10 SnRK2 (Fig. 3C). It should be noted that two forms of SnRK2-8 were produced in bacteria, both recognized by the antibody. Moreover, the antibody failed to recognize any of the 10 recombinant proteins of an unrelated kinase family, the AtSK (data not shown). Taken together, these results indicate that the anti-SnRK2 antibody is specific to the SnRK2 family. Using the anti-SnRK2 antibody in immunoprecipitation followed by gel kinase assay (Fig. 4), a strong activation by hyperosmolarity was observed for four SnRK2 with the same apparent molecular masses as in crude extracts (35, 37–38, and 42 kDa). Like in crude extracts, the activations were stronger with the increased stress of osmolarity (500 and 1000 mM sucrose). The specificity of the immunoprecipitation was shown using successfully the SnRK2 peptide as a competitor, whereas another unrelated peptide (AtSK) had no effect. Moreover, the 44-kDa kinase corresponding to AtMPK6 (1) is not immunoprecipitated by anti-SnRK2 antibody. It should be noted that the SnRK2 antibody revealed four activity bands (Fig. 4), whereas only three of them were detected by immunoblotting on total extracts (Fig. 3B). This could be explained by different expression levels, various specific activities, and/or activation intensities from one SnRK2 to another. Nevertheless, the results obtained using the family-specific antibody indicate that at least four SnRK2 kinases are activated by hyperosmolarity in Arabidopsis cell suspension.

Identification of Nine SnRK2 Kinases Activated by Hyperosmolarity—The identification at the molecular level of the hyperosmotically activated SnRK2 was investigated using a transient expression assay. Arabidopsis protoplasts can be efficiently transformed but, lacking cell wall, they can have lost some transduction components. Thus, we first checked by MBP in-gel kinase assay that SnRK2 kinases were also activated by

**Fig. 1.** Several protein kinases are activated by hyperosmolarity in Arabidopsis cells and seedlings, whatever the osmolyte used. Kinase activity was determined by in-gel kinase assay using MBP or histone as substrates. A, equilibrated cells were transferred to isoosmotic medium (iso), moderate hyperosmotic medium (hyper 500), or high hyperosmotic medium (hyper 1000, 1 M sucrose) for 10 min. B, cells were treated as described in A. High hyperosmotic stress was induced by 1 M sucrose (suc), 1 M mannitol (man), or 650 mM NaCl (NaCl). C, seedlings grown on control medium for 6 days were transferred to isoosmotic medium (iso), moderate hyperosmotic medium (hyper 500, 500 mM sucrose), or high hyperosmotic medium (hyper 1000, 1 M sucrose) for 30 min.

When Arabidopsis seedlings were submitted to moderate and high hyperosmotic stresses, several protein kinases were activated with profiles comparable with those observed in cells (Fig. 1C). Using MBP as a substrate, only the 42-, 38-, and 37-kDa activity bands were visualized, whereas on histone, the four non-MAPK protein kinases were detected. Unlike in cells, kinase activations hardly increased in plantlets submitted to high hyperosmolarity in comparison with moderate stress. This result suggests that signaling induced by high hyperosmotic medium on seedlings (1 M sucrose) corresponds only to signaling induced by a moderate hyperosmotic condition on cell suspension (500 mM sucrose). Coherently, the 44 kDa displayed a strong activation in plantlets exposed to high stress, whereas the activity of this kinase decreased in cell suspension when the stress strength increased. As in cells, the 44-kDa kinase corresponds to AtMPK6 (data not shown). Thus, the four non-MAPK protein kinases are activated both in cells and seedlings, confirming that Arabidopsis cell suspension is a suitable model to study these kinases in response to osmotic stresses.

**The Protein Kinases Activated by Hyperosmolarity in Arabidopsis Cells Are SnRK2**—The four non-MAPK protein kinases activated by hyperosmotic stresses in cells and seedlings remained to be identified. It was assumed that they could belong to the Arabidopsis SnRK2 family and the expression of the 10 members of the family was first evaluated (Fig. 2). Using RT-PCR, the presence of SnRK2 transcripts was checked in cells and seedlings at the same physiological stages used for in-gel kinase assays. In these conditions, the 10 members of the SnRK2 family are expressed both in cells and seedlings, making this kinase family a suitable candidate. To search for the possible involvement of SnRK2 kinases in osmotic response, an SnRK2 family-specific antibody raised against a conserve peptide was prepared. The peptide used for immunization was chosen in the catalytic domain, between VII and VIII subdomains (Fig. 3A). The quality of the antibody was checked by immunoblotting on total proteins from cell suspension (Fig. 3B). The anti-SnRK2 antibody recognized three major bands with apparent molecular masses of 42, 38, and 37 kDa, which are close to the predicted molecular masses of SnRK2 proteins (from 38 to 43 kDa). It should be noted that some faint additional bands can also be visualized that probably correspond to unspecific background and/or degradation products. To further characterize the antibody, its ability to recognize the 10 SnRK2 proteins was analyzed by immunoblotting on recombinant proteins. Each SnRK2 kinase was produced in bacteria with a His-tag and purified on nickel column. The recombinant proteins were immunoblotted with the anti-SnRK2 antibody that succeeded in recognizing the 10 SnRK2 (Fig. 3C). It should be noted that two forms of SnRK2-8 were produced in bacteria, both recognized by the antibody. Moreover, the antibody failed to recognize any of the 10 recombinant proteins of an unrelated kinase family, the AtSK (data not shown). Taken together, these results indicate that the anti-SnRK2 antibody is specific to the SnRK2 family. Using the anti-SnRK2 antibody in immunoprecipitation followed by in-gel kinase assay (Fig. 4), a strong activation by hyperosmolarity was observed for four SnRK2 with the same apparent molecular masses as in crude extracts (35, 37–38, and 42 kDa). Like in crude extracts, the activations were stronger with the increased stress of osmolarity (500 and 1000 mM sucrose). The specificity of the immunoprecipitation was shown using successfully the SnRK2 peptide as a competitor, whereas another unrelated peptide (AtSK) had no effect. Moreover, the 44-kDa kinase corresponding to AtMPK6 (1) is not immunoprecipitated by anti-SnRK2 antibody. It should be noted that the SnRK2 antibody revealed four activity bands (Fig. 4), whereas only three of them were detected by immunoblotting on total extracts (Fig. 3B). This could be explained by different expression levels, various specific activities, and/or activation intensities from one SnRK2 to another. Nevertheless, the results obtained using the family-specific antibody indicate that at least four SnRK2 kinases are activated by hyperosmolarity in Arabidopsis cell suspension.
SnRK2-6
SnRK2-8
SnRK2-3
SnRK2-1
SnRK2-5
SnRK2-4
SnRK2-10
SnRK2-9
SnRK2-7

VII

PAK1T1CDGYYGSQKSVLHST3
TVRYTGAIQIAEVLY

VIII

PAK1T1CDGYYGSQKSVLHST3
TVRYTGAIQIAEVLY

B

Coomassie blue

Immunoblot

kDa 1 2 3 4 5 6 7 8 9 10

41

41

42

42

35

38

37

50

75

100

150

250

M c.e. kDa

FIG. 3. The anti-SnRK2 antibody specifically recognizes the 10 SnRK2 proteins. A, amino acid sequence alignment of the SnRK2 VII and VIII catalytic subdomains was performed with CLUSTALX algorithm. Identical residues are boxed in black and similar residues are boxed in gray. The sequence of the peptide used for immunization corresponds to SnRK2-4 and is indicated by the black line. B, crude extract from cell suspension (c.e.) was separated by SDS-PAGE and immunoblotted with anti-SnRK2 antibody. Molecular masses of the markers (M) are indicated on the left, whereas apparent molecular masses of the recognized proteins are indicated on the right. C, Histagged SnRK2 proteins were produced in E. coli and purified on nickel columns. They were separated by SDS-PAGE and immunoblotted with anti-SnRK2 antibody (upper panel) or stained with Coomassie Blue (lower panel). The mean of the apparent molecular masses of the kinases (41 kDa) is indicated on the right.

Activation by Hyperosmotic Stresses of Arabidopsis SnRK2

hyperosmolarity in Arabidopsis protoplasts. Untransfected protoplasts were transfected to isosmotic medium (400 mM) or mannitol high hyperosmotic medium (1000 mM) as for cells. In such conditions, several kinases were activated in crude extract with the same profile as in cells (Fig. 5A). Using the anti-SnRK2 antibody in immunoprecipitation followed by in-gel kinase assay, a strong activation of the four SnRK2 bands (35-, 37-, 38-, and 42-kDa) was observed in hyperosmotic condition (Fig. 5A).

Protoplasts being a suitable model to identify the activated SnRK2, Arabidopsis protoplasts were transiently transformed with an expression vector for each HA-tagged SnRK2 or the empty vector as a control. Transfected protoplasts were treated for mannitol high hyperosmotic stress as described above. After immunoprecipitation with anti-HA antibody, SnRK2 activity was determined by in-gel kinase assay using MBP as a substrate. The expression level of each HA-tagged SnRK2 was monitored by immunoblotting with anti-HA antibody (Fig. 5B). Surprisingly, all SnRK2 except SnRK2-9 were activated by hyperosmotic stress. Three activation levels by hyperosmolarity can be distinguished: a very strong activation of SnRK2-6, a strong activation of SnRK2-2, SnRK2-3, SnRK2-8, and SnRK2-10, and a lower activation of SnRK2-1, SnRK2-4, SnRK2-5, and SnRK2-7.

SnRK2 Kinases Are Differentially Activated by Salt Stress and ABA but Not by Cold—Transduction pathways of environmental stresses appear very complex, with crosstalks between osmotic, salt, and ABA signaling (38). As nine of 10 SnRK2 kinases are activated by hyperosmotic stress, the possible involvement of these kinases in other signal transduction pathways was investigated. As for hyperosmotic stress, the activation of SnRK2 kinases by salt stress, cold, or ABA treatments was first tested in untransfected protoplasts with the family-specific antibody. For salt stress, protoplasts were transferred to isosmotic medium (400 mM) or NaCl hyperosmotic medium (1000 mM). For cold treatment, protoplasts were incubated at 4°C or at room temperature as a control. For ABA treatment, protoplasts were incubated with 30 μM ABA or the same volume of ethanol solvent control. Using the anti-SnRK2 antibody in immunoprecipitation followed by in-gel kinase assay (Fig. 6A), four SnRK2 of 35, 37, 38, and 42 kDa were strongly activated by salt stress, as observed with mannitol hyperosmotic stress, whereas ABA only activated SnRK2 of 38 and 42 kDa and more slightly a 35-kDa kinase. On the other hand, cold treatment did not activate any SnRK2 kinase even when the stress duration was increased to 30 min (data not shown).

As different SnRK2 were activated by salt stress and ABA, the transient expression assay was performed to identify the SnRK2 activated by each signal. Salt stress and ABA treatment were performed as described above. SnRK2 activity after immunoprecipitation and expression levels of each tagged kinase were analyzed as previously. Salt stress activated the same kinases as mannitol and with the same intensity, except for SnRK2-10, which is more weakly activated by NaCl than mannitol (Fig. 6B). This result is consistent with the identical kinase activation profile observed in response to sucrose, mannitol, and NaCl in Arabidopsis cell suspension (Fig. 1B). It was noted that SnRK2-1 and SnRK2-5 sometimes display a doublet, depending on the experiment. By contrast with hyperosmotic and saline treatments, ABA only activated five SnRK2 (Fig. 6C). As for mannitol treatment, a very strong activation of SnRK2-6, a strong activation of SnRK2-2 and SnRK2-3, and a lower activation of SnRK2-7 were observed. However, SnRK2-8 was only very slightly activated by ABA, contrary to the high activation by hyperosmotic stress previously observed.

DISCUSSION

Osmotic signaling involves different protein kinase families and among them the MAPK family is the best characterized in
plants. Here we describe the activation by hyperosmotic stresses of several Arabidopsis Ca\textsuperscript{2+}-independent protein kinases with apparent molecular masses of 35, 37, 38, and 42 kDa. These kinases can use both MBP and histone as substrates (Fig. 1A) and it was previously shown that their activation was insensitive to apigenin, a MAPK inhibitor, and to a protein-tyrosine phosphatase treatment (1). Taken together, these results indicate that hyperosmotic stress activates at least four protein kinases that do not belong to the MAPK family in Arabidopsis cell suspension. Moreover, activations were induced by different osmolytes such as sucrose, mannitol, and NaCl (Fig. 1B), and occurred from 2 min of stress (1),

**Fig. 5. All SnRK2 kinases except SnRK2-9 are activated by hyperosmolality.** A, protoplasts were transferred to isoosmotic medium (400 mOsm (−)) or mannitol hyperosmotic medium (1000 mOsm (+)) for 10 min. Kinase activity of the crude extracts or of the SnRK2 immunocomplexes was performed with an MBP in-gel kinase assay. B, Arabidopsis protoplasts were transiently transformed with the empty vector (−) or the expression vector for each SnRK2. Osmotic stress was performed as in A. HA-tagged SnRK2 proteins were immunoprecipitated from protein extracts with anti-HA antibody and analyzed by in-gel kinase assay using MBP as a substrate (upper panel). The expression of each kinase was monitored by immunoblotting the same extracts with anti-HA antibody (lower panel).

**Fig. 6. The SnRK2 kinases are differentially activated by NaCl and ABA, but not by cold.** A, protoplasts were transferred to isoosmotic medium (400 mOsm (−)) or NaCl hyperosmotic medium (1000 mOsm (+)) for 10 min (left panel). Protoplasts were incubated at room temperature (−) or at 4 °C (+) for 10 min (center panel). Protoplasts were treated with 30 μM ABA (+) or the same volume of ethanol solvent control (−) for 20 min (right panel). Kinase activity of the crude extracts or of the SnRK2 immunocomplexes was performed with an MBP in-gel kinase assay. B and C, Arabidopsis protoplasts were transiently transformed with the empty vector (−) or the expression vector for each SnRK2. Salt (B) and ABA (C) treatments were performed as in A. Immunoprecipitation followed by in-gel kinase assay (upper panel) and immunoblot analysis (lower panel) were performed as described in the legend to Fig. 5.
indicating that these kinases are involved in a general and early response to osmotic stress.

Similar kinase activation profiles were observed when cell suspension, seedlings (Fig. 1, A and C), or protoplasts (Fig. 5A) were exposed to hyperosmolarity, indicating that cells and protoplasts may reflect physiological response in plants, at least for the early transduction steps. However, it should be noted that electrophoretic migration of the kinases was slightly different depending on plant material, which could be because of differences in the composition of protein extracts. The kinase activation was also slightly delayed in plantlets in comparison to cell suspension. Moreover, the intensity of kinase activation looked similar in cell suspensions submitted to moderate stress and seedlings exposed to high stress (Fig. 1C). This difference between cells and plant tissues was also reported for the phosphorylation of the phosphatidylinositol transfer protein SSH1 induced by the hyperosmotic condition in tobacco (32). The activation occurred for a weaker stress strength in cells in comparison to leaf tissue and the authors explained this result by the difficulty to apply a uniform osmotic stress to all cells when using intact tissue. Thus, cell suspension and protoplasts appear to be suitable models to study early plant osmotic responses.

Based on the activation of one SnRK2 by hyperosmolarity in tobacco cells (4), the non-MAPK protein kinases visualized here in Arabidopsis were suggested to belong to the SnRK2 family. Only little is known about the members of this family, either on the expression pattern or physiological roles (see Ref. 29 for review). The 10 Arabidopsis SnRK2 were expressed both in cells and seedlings (Fig. 2), and thus the whole kinase family constituted a suitable candidate. To investigate the involvement of SnRK2 in osmotic signaling, a family-specific antibody was prepared. Immunoblot analysis with recombinant proteins showed that the antibody could recognize the 10 SnRK2 proteins (Fig. 3C) but none of the 10 unrelated AtSK. Moreover, the antibody only recognized three bands on the total protein extract from cell suspension (Fig. 3B), with apparent molecular masses (37, 38, and 42 kDa) corresponding to SnRK2 in size, indicating that the antibody was specific to the SnRK2 family. Using this antibody in immunoprecipitation coupled with in-gel kinase assay, the activation of at least four SnRK2 was visualized (Fig. 4). The SnRK2 bands displayed the same molecular masses as those observed in crude extracts, suggesting that they are the same kinases. This is the first demonstration of activation by hyperosmotic stresses of several SnRK2 in native conditions, indicating an important role of this family in osmotic signaling.

To identify the SnRK2 kinases at the molecular level, a transient expression assay was performed. Using a 35 S promoter, the overexpression of the kinases could interfere with natural signal transduction pathways and not reflect physiological responses. However, the expression of the tagged kinases remains moderate because they are hardly detectable by the highly sensitive SYPRO Ruby staining (Molecular Probes, data not shown). In the transient expression assay, mannitol hyperosmotic stress activated nine of the 10 SnRK2 kinases (Fig. 5B). It could be surprising that nine SnRK2 were activated, whereas only four activity bands were observed in crude extracts. However, SnRK2 kinases have very similar predicted molecular masses, which is correlated with similar electrophoretic migration on SDS-PAGE, as seen in immunoblot analysis (Fig. 5B, lower panel). Thus, based on apparent molecular masses, a correspondence between activity bands in crude extracts and SnRK2 proteins can be established. The higher 42-kDa band would correspond to SnRK2-6, whereas the lower 35-kDa band may regroup SnRK2-1 and SnRK2-8. The other activated SnRK2 (SnRK2-2, SnRK2-3, SnRK2-4, SnRK2-5, SnRK2-7, and SnRK2-10) would constitute the 37–38-kDa thick band. SnRK2 were hyperosmotically activated with three different activation intensities, suggesting that they may play a specific role according to their activation level. These results are consistent with other published data that describe activation of SnRK2. The tobacco ASK1 homolog activated by hyperosmotic stress (4) and the Arabidopsis SnRK2-E responsive to dehydration (30) correspond to SnRK2-4 and SnRK2-6, respectively. Coherently, these two kinases are shown here to be activated by hyperosmotic and saline stresses. In soybean, Monks et al. (32) described the activation of only two of four SnRK2 (SPK1 and SPK2) in yeast exposed to high hyperosmolarity. In the meantime, Kobayashi et al. (39) described the salt activation of the 10 rice SnRK2 by transient expression assays. Two of them (SAPK1 and SAPK2) were also shown to be activated by mannitol but not by cold. In the present study, the activation of the whole family members by different osmotic signals was studied in Arabidopsis. Coherently with the result concerning SAPK1 and SAPK2, cold treatment did not activate any of the 10 Arabidopsis SnRK2 (Fig. 6A, center panel), whereas salt stress activated the same nine SnRK2 than mannitol stress (Fig. 6B), confirming that SnRK2 are activated in a general osmotic response as observed in cells (Fig. 1B).

SnRK2-9, which was the only SnRK2 not to be activated by any of the four signals tested (mannitol, NaCl, cold, and ABA), is the most divergent protein of the family (Fig. 7A). Even though SnRK2-9 displayed weaker expression in protoplasts than the other SnRK2 (Figs. 5B and 6, B and C, lower panels), the non-activation cannot be explained by a lower protein

![Fig. 7. Arabidopsis SnRK2 family and its involvement in osmotic signaling.](image-url)
amount because the other SnRK2 retained activation even when expressed lower than SnRK2-9 (data not shown). Thus SnRK2-9 may be involved in another signal transduction pathway and information on its protein expression in plant should provide some indications.

Interestingly, ABA only activated a part of the nine SnRK2 (Fig. 6C) but again with distinct activation levels. So, in the same overexpression conditions, the kinases are differentially activated by distinct stresses (osmotic shocks, cold, and ABA treatment), confirming the validity of the approach. However, it cannot be excluded that activation levels observed in transient expression assays may slightly differ from the corresponding levels in planta. Based on ABA activation, SnRK2 can be divided in two subgroups (Fig. 7A): one group composed of kinases activated by both ABA and hyperosmotic stresses, including SnRK2-2, SnRK2-3, SnRK2-6, SnRK2-7, and SnRK2-8 (group 1), and the other one composed of kinases only activated by hyperosmotic conditions, including SnRK2-1, SnRK2-4, SnRK2-5, and SnRK2-10 (group 2). This classification is coherent with the presence in group 1 of the barley PKABA1, which was transcriptionally induced by ABA (40) and the Vicia faba AAKP, which was activated by ABA in guard cell protoplasts (41, 42). Moreover, SnRK2-6/SnRK2-E/OST1 was demonstrated to be activated in an ABA-dependent signaling pathway leading to stomatal closure (30, 43), suggesting that SnRK2-6 is also involved in an ABA-dependent osmotic pathway. However, further experiments using ABA-deficient or -insensitive mutants are needed to conclude on the ABA dependence of group 1 SnRK2 activation.

Considering the present work and already quoted studies, a schematic summary of the different protein kinase families involved in Arabidopsis osmotic signaling is proposed, which attempts to include ABA and calcium signals (Fig. 7B). Although calcium signals elicited by hyperosmotic stresses, cold, and drought have been widely described, little is known about the downstream targets (21). For instance, whereas many works reported the stress activation of MAPK, only three studies investigated the role of calcium. The hyperosmotic activation of AtMPK6 (1) and the cold activation of SAMK (44) required calcium influx, whereas SIPK activation by hyperosmotic stresses was Ca\(^{2+}\)-independent (5). Concerning SnRK2, we have previously shown that EGTA and gadolinium, a calcium channel inhibitor, had no effect on the activation of the non-MAPK protein kinases in Arabidopsis cells submitted to hyperosmolality (1), suggesting that SnRK2 activation is Ca\(^{2+}\)-independent. However, we demonstrated in the present study that these four activity bands corresponded to nine SnRK2, making interpretation more difficult. So it cannot be excluded that calcium is required for the activation of some SnRK2. Fig. 7B clearly illustrates cross-talks between hyperosmotic stress, NaCl, drought, and cold at the kinase level, but also the existence of pathways specific to each stress. Downstream responses including gene expression regulation also underline interactions between signaling pathways but links between upstream kinases and downstream targets are lacking (45, 46).

As nine SnRK2 are activated by hyperosmotic stresses, it would be interesting to determine whether they act in the same pathway. It was shown that SnRK2 displayed different activation intensities and only five of the nine kinases were also activated by ABA, suggesting that they could be involved in several distinct signaling pathways. To go further, analysis of downstream elements like gene expression in snrk2 mutants may provide evidence about the common and distinct components of the pathways. Likewise, it would be interesting to search for upstream elements and partners. On this respect, determining the cellular location of the kinases could give a first indication. Although no data is available on their possible location, the absence of peptide signal in their sequence suggests that they are likely to be cytoplasmic. Further experiments are needed to specify the cellular location of SnRK2 kinases, and whether they move in the cell after stresses to reach their targets. On the other hand, one common kinase activation process is phosphorylation by upstream kinases. Concerning SnRK2, divergent results were obtained using treatments by phosphatase or inhibitors of phosphatase. The tobacco ASK1 homolog (4) and two rice SnRK2 (39) were shown to be activated by phosphorylation. In contrast, Hoyos and Zhang (5) reported activation by hyperosmolality of an unknown kinase HOSAK, which is likely to also be an SnRK2, through a dephosphorylation process. This question is now under investigation in the laboratory for the whole Arabidopsis SnRK2 family.

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Identification of Nine Sucrose Nonfermenting 1-related Protein Kinases 2 Activated by Hyperosmotic and Saline Stresses in Arabidopsis thaliana
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