Arabidopsis decuple mutant reveals the importance of SnRK2 kinases in osmotic stress responses in vivo

Hiroaki Fujii[a,b], Paul E. Verslues[a,1], and Jian-Kang Zhu[a,b,c,2]

[a]Department of Botany and Plant Sciences, University of California, Riverside, CA 92521; [b]Center for Plant Stress Genomics and Technology, King Abdullah University of Science and Technology, Thuwal 23955-6900, Kingdom of Saudi Arabia; and [c]Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907-2010

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Osmotic stress associated with drought or salinity is a major factor that limits plant productivity. Protein kinases in the SNF1-related protein kinase 2 (SnRK2) family are activated by osmotic stress, suggesting that the kinases are involved in osmotic stress signaling. However, due to functional redundancy, their contribution to osmotic stress responses remained unclear. In this report, we constructed an *Arabidopsis* line carrying mutations in all 10 members of the SnRK2 family. The decuple mutant *snrk2.1/2/3/4/5/6/7/8/9/10* grew poorly under hyperosmotic stress conditions but was similar to the wild type in culture media in the absence of osmotic stress. The mutant was also defective in gene regulation and the accumulation of abscisic acid (ABA), proline, and inositol 1,4,5-trisphosphate under osmotic stress. In addition, analysis of mutants defective in the ABA-activated SnRK2s (*snrk2.2/3/6*) and mutants defective in the rest of the SnRK2s (*snrk2.1/4/5/7/8/9/10*) revealed that SnRK2s are a merging point of ABA-dependent and -independent pathways for osmotic stress responses. These results demonstrate critical functions of the SnRK2s in mediating osmotic stress signaling and tolerance.

abiotic stress | water deficit | osmosensing | signal transduction | phosphorylation

Drought and soil salinity are major environmental factors limiting plant agricultural productivity. Both drought and salinity cause osmotic stress to plants. Osmotic stress activates signaling pathways that lead to rapid changes in gene expression and metabolism, some of which presumably help plants to cope with the stress. One important response to osmotic stress is the accumulation of the stress hormone abscisic acid (ABA). ABA is required for plant resistance to drought or salt stress (1–6). In the cytoplasm and nucleus, ABA binds to the PYR/PYL/RCAR family of START domain-containing soluble receptors (7, 8). The binding causes the receptors to bind and inhibit the activities of the type A protein phosphatase 2Cs (PP2Cs). Consequently, the PP2Cs lose their ability to interact with and inhibit the subfamily of SNF1-related protein kinase 2s (SnRK2s) such as SnRK2.2, SnRK2.3, and SnRK2.6 (also known as OST1 or SRK2E; refs. 9–11). The activated SnRK2s can then phosphorylate downstream proteins such as ABA-responsive element (ABRE)-binding transcription factors (ABFs; refs. 12–14) and Slow Anion Channel 1 (SLAC1; refs. 15 and 16), NADPH oxidase (17), and a potassium channel (18) to activate ABA-responsive gene expression and cause stomatal closing. In contrast to the recent, rapid progress in our understanding of the ABA signaling pathway, little is known about the signaling pathway that leads from osmotic stress sensing to the activation of ABA accumulation. ABA accumulation under osmotic stress results from increased biosynthesis and reduced catabolism (19). The biosynthesis genes are induced or upregulated by osmotic stress (20, 21).

The SnRK2 protein kinase family consists of 10 members (SnRK2.1 to -2.10) in *Arabidopsis* and 10 (SAPK1 to -10) in *Oryza sativa* (22). Several SnRK2s have been reported to be major kinases in ABA signaling, which regulates various developmental and physiological processes including seed maturation, dormancy, seedling development, and stomatal behavior. The first report of SnRK2 involvement in ABA signaling was for the wheat SnRK2-type protein kinase PKABA1, which acts downstream of ABA during germination and is induced by ABA at the transcript level (23). The *Vicia faba* SnRK2-type protein kinase AAPK is activated by ABA in guard cells and regulates stomatal closure (24). In *Arabidopsis*, the *snrk2.6* (*ost1*) mutant cannot exhibit ABA-induced stomatal closure (25, 26), and the *snrk2.2/2.3* double mutant is insensitive to ABA in terms of seed germination and seedling growth (27). In the *snrk2.2/3/6* triple mutant all examined ABA responses are blocked (28–30). Thus, SnRK2.2, -2.3, and -2.6 act redundantly in ABA regulation of guard cells, seed germination and seedling growth.

In vitro assays have shown that SnRK2.2, -2.3, and -2.6 are strongly activated by ABA, whereas SnRK2.7 and -2.8 are weakly activated by ABA (13, 31). Interestingly, all of the SnRK2s except SnR2.9 can be activated by osmotic stress in *Arabidopsis* protoplasts (31). Activation of SnRK2 homologs was also observed in tobacco (32), soybean (33), and rice (34). The osmotic stress activation of the SnRK2s suggests that the kinases play roles in osmotic stress signaling. However, the physiological role of SnRK2s in osmotic stress responses has remained unclear. The extreme-drought stress sensitivity of the *snrk2.2/3/6* triple mutant may be explained by defective guard-cell regulation due to ABA insensitivity (28, 29). It is unclear whether these kinases may also have a direct role in osmotic stress signaling. Although root growth of the *snrk2.8* mutant on agar plates has increased sensitivity to drought and overexpression of SnRK2.8 or the wheat TaSnRK2.4 improved drought tolerance (35, 36), drought tolerance in soil was similar among the wild type, *snrk2.7*, *snrk2.8*, and the *snrk2.7/2.8* double mutant (37). The difficulty in defining the physiological roles of the SnRK2s in osmotic stress signaling and tolerance is likely a result of a high degree of functional redundancy among these genes. According to published microarray data (genevestigator: https://www.genevestigator.com/gv/index.jsp), the expression of all ten members of SnRK2 genes is detectable in most tissues. To determine whether redundancy masks defects caused by mutations in some of the SnRK2s, we constructed a decuple mutant carrying loss-of-function mutations in all 10 members of the SnRK2 family in *Arabidopsis*. This decuple mutant and other combinations of *snrk2* mutants allowed us to directly determine a role for these genes in osmotic stress signaling and tolerance.

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[1]Present address: Institute of Plant and Microbial Biology, Academia Sinica, Taipei 11529, Taiwan.

[2]To whom correspondence should be addressed. E-mail: jkzhu@purdue.edu.

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Results

Construction of the *snrk2.1/2/3/4/5/6/7/8/9/10* Decuple Mutant. To generate the decuple mutant *snrk2.1/2/3/4/5/6/7/8/9/10*, we crossed *snrk2* T-DNA mutants (Fig. S1*A*) sequentially and produced a mutant homozygous for *snrk2.1/3/4/5/6/7/8/9/10* but heterozygous for *snrk2.2* (herein after referred to as 2hetero). We also identified the *snrk2.1/4/5/7/8/9/10* septuple mutant (herein after referred to as the septuple) and the *snrk2.1/3/4/5/6/7/8/9/10* nonuple mutant, in which only *SnRK2.2* is of the wild-type allele (herein after referred to as 2W).

To identify a decuple mutant among the progenies of 2hetero, seeds were screened on MS agar plates containing 5 μM ABA because the *snrk2.2/3/6* triple mutant is insensitive to ABA in seed germination (28). Germinated seeds were confirmed to be the decuple mutant by genomic PCR, and RT-PCR data confirmed that the decuple mutant did not express any intact *SnRK2* mRNAs (Fig. 1). Typically <5% of the seeds germinated on the ABA plate rather than the expected 25% homozygous for *snrk2.2*; this is likely because *snrk2.2/3/6* triple mutant seeds frequently did not mature (28). Thus, many of the decuple mutant seeds also failed to mature, resulting in <25% germination rate. Consistent with this result, siliques of 2hetero plants contained aborted seeds (Fig. S2*A*).

Because the *snrk2.2/3/6* triple mutant has a wilty phenotype (28), the decuple mutant plants grown in soil were covered with plastic lids to maintain high humidity (96–100% relative humidity). Under these high-humidity conditions, the decuple mutant plants could grow, bolt, and flower, but were much smaller than the *snrk2.2/3/6* triple mutants, which were also smaller than the wild type (Fig. 1*B*). In addition, the decuple mutant failed to produce seeds even though it produced flowers with a pistil and stamens (Fig. S2*B*).

Decuple Mutant Is Deficient in Osmotic Stress-Activated Protein Kinase Activity. The activation status of SnRK2s in vivo can be detected by in-gel kinase assays with histone as the substrate. Such assays using *Arabidopsis* crude extracts have revealed that osmotic stress activates several protein kinases (26, 31, 38). We performed in-gel kinase assays using extracts from plants exposed to osmotic stress in liquid culture. Four bands could be detected in extracts from wild-type plants under untreated control conditions (Fig. 2*A*). The two bands around 40–42 kDa were probably from activities of SnRK2s in the SnRK2.1/4/5/7/8/9/10 group because they were reduced or disappeared in the septuple and decuple mutants. The two larger bands (around 48 and 60 kDa) were outside the size range of the SnRK2s and did not change in the decuple mutant, and thus were clearly not from SnRK2s. In extracts from wild type treated with 0.8 M mannitol for 20 min, at least three bands around 42 kDa were activated (Fig. 2 *A* and *B*). According to previous experiments (27), SnRK2.2 is included in the upper band. In the *snrk2.1/4/5/7/8/9/10* septuple mutants, the lower and middle bands almost disappeared, but the upper band remained. In the decuple mutant, the remaining upper band also disappeared (Fig. 2 *A* and *B*). These results indicate that the mannitol-activated bands in the in-gel kinase assay are SnRK2s and that SnRK2 activities were eliminated in the decuple mutant.

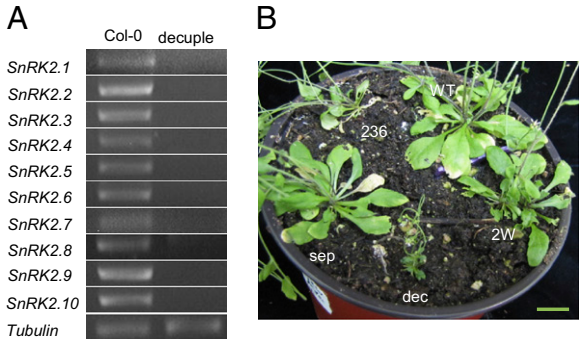

Fig. 1. Isolation of the decuple mutant. (*A*) RT-PCR analysis with *SnRK2s* and *Tubulin* primers using total RNA extracted from seedlings of the wild type (Col-0) and the decuple mutant as the template. (*B*) Photograph of wild type (WT) and *snrk2.2/3/6* (236), *snrk2.1/4/5/7/8/9/10* septuple (sep), *snrk2.1/2/3/4/5/6/7/8/9/10* decuple (dec), and *snrk2.1/3/4/5/6/7/8/9/10* nonuple (2W) mutant plants. The plants were grown in soil for 4 wk after transfer from MS plates on which the plants had germinated and had been identified. (Scale bars: 1 cm.)

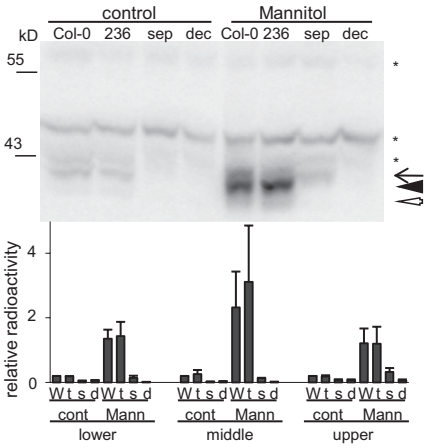

Fig. 2. In-gel kinase assay. In-gel kinase assay with proteins extracted from the wild type (Col-0) and *snrk2.2/3/6* (*236*), *snrk2.1/4/5/7/8/9/10* septuple (sep), and *snrk2.1/2/3/4/5/6/7/8/9/10* decuple (dec) mutants grown in liquid culture with or without a 20-min exposure to 0.8 M mannitol. Histone was used as the substrate. In the autoradiograph (*Upper*), the upper, middle, and lower activated bands are indicated by an arrow, a closed arrowhead, and an open arrowhead, respectively. Asterisks indicate noninduced bands. (*Lower*) Relative radioactivity (mean ± SEM, $n = 3$) of the lower, middle, and upper bands with mannitol (mann) or without mannitol (cont) normalized relative to band activity of the wild type without mannitol. Col-0 and the *snrk2.2/3/6* triple, the septuple, and the decuple mutants are indicated by W, t, s, and d, respectively.

Decuple Mutant Is Hypersensitive to Osmotic Stress. We examined the effect of lack of SnRK2s on seedling growth under osmotic stress conditions. Because the decuple mutant did not produce seeds, obtaining plants of the decuple mutant required repeated screening of 2hetero progeny. The decuple mutants identified on 5 μM ABA plates were transferred to 1/2 MS plates without ABA for recovery by further incubation for 5 d. The seedlings were subsequently transferred to control plates or plates containing a low concentration of PEG (−0.4 MPa; ref. 39). Similar sized seedlings of the wild type, *snrk2.2/3/6*, and the septuple mutant were also transferred to control or PEG plates. Although growth of all of the genotypes was similar on control plates, growth of the decuple mutant, but not growth of the wild type, was severely inhibited on PEG medium (Fig. 3*A*). In addition, leaves of the decuple mutant seedlings turned yellow under PEG treatment (Fig. 3*A*). The sensitivity of *snrk2.2/3/6* and the septuple mutant to PEG was variable; some seedlings grew well, whereas others grew poorly. Quantitative data showed that PEG greatly reduced the fresh weight and root growth of the decuple mutant but only moderately reduced the fresh weight and root growth of *snrk2.2/3/6* and the septuple mutant (Fig. 3*B*). Compared with the *snrk2.2/3/6* triple mutant, the septuple mutant was more affected by osmotic stress and had less fresh weight. The growth of the 2W nonuple mutant was similar to that of the

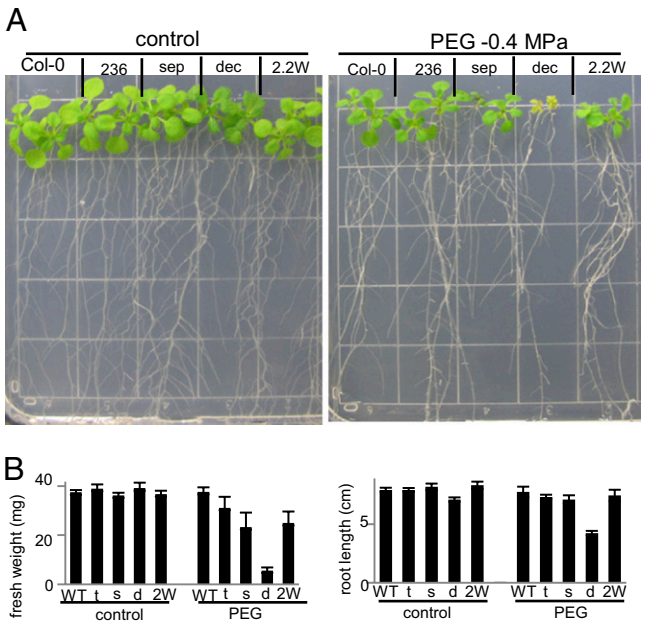

Fig. 3. Seedling growth under osmotic stress. (*A*) Photographs of seedlings 18 d after transfer to the control medium (1/2 MS/1% sucrose) or medium containing PEG (−0.4 MPa). (*B*) Seedling fresh weight and root length for seedlings treated as in *A*. For fresh weight determination, two seedlings were weighed at one time, and the weight was divided by two. Values are means ± SEM (n = 5 for fresh weight and 10 for root length). Col-0 and the *snrk2.2/3/6* triple, the septuple, the decuple, and the nonuple mutants are indicated by WT, t, s, d, and 2W, respectively.

septuple mutant (Fig. 3*B*). These results indicate that SnRK2s are required for growth under osmotic stress conditions and that there is high redundancy because the presence of SnRK2.2 alone could largely rescue the decuple mutant phenotype.

Decuple Mutant Is Impaired in Osmotic Stress-Induced ABA Accumulation. We analyzed ABA and H_2O_2 contents in the mutant plants under osmotic stress. After a 12-h exposure to media containing PEG (−1.2 MPa), ABA increased by ∼10- to 17-fold in the wild type, *snrk2.2/3/6* triple mutant, and septuple mutants. However, the ABA content increased by only about twofold in the decuple mutant in response to the same PEG treatment (Fig. 4*A*). These results suggest that the SnRK2s are critical for ABA accumulation under osmotic stress.

Some mutants that are sensitive to osmotic or salt stress accumulate more reactive oxygen species (ROS; ref. 40). ROS may cause cell damage, even though ROS at low levels are also used as signaling molecules. We examined the possibility that the growth defect of the decuple mutant under osmotic stress resulted from ROS accumulation as indicated by H_2O_2 concentration. After a 12-h exposure to medium containing PEG (−1.2 MPa), H_2O_2 contents increased in all mutants and in the wild type. The H_2O_2 concentration was similar in the decuple mutant and the wild type (Fig. 4*B*). These results suggest that the phenotype of the decuple mutant under osmotic stress is not a result of ROS over accumulation.

Decuple Mutant Is Defective in Osmotic Stress Induction of Gene Expression. Osmotic stress induces the expression of many genes in *Arabidopsis* (41). To examine the effect of *snrk2* mutants on osmotic stress-induced gene expression, we performed quantitative RT-PCR (qPCR) of several known osmotic-stress-induced genes (41). In the wild type, expression of *RD29A*, *NCED3*, *RD26*, and *PKS5* was induced in seedlings grown in liquid culture

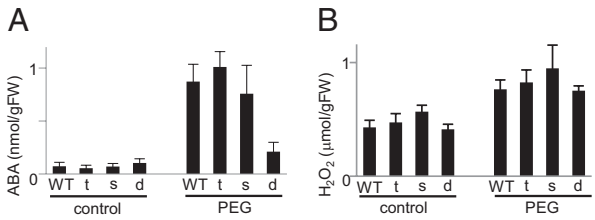

Fig. 4. ABA and H_2O_2 contents of the mutants under osmotic stress. ABA (*A*) and H_2O_2 (*B*) contents were measured in the wild type (WT) and in the snrk2.2/3/6 triple (t), the septuple (s), and the decuple (d) mutants 12 h after transfer to control medium (1/2 MS/1% sucrose) or to medium containing PEG (−1.2 MPa). Values are means ± SEM (n = 3).

after 3 h of treatment with 0.8 M mannitol. Induction of these genes was substantially reduced in the decuple mutant and in the *snrk2.2/3/6* triple mutant. Effect of the *snrk2.1/4/5/7/8/9/10* septuple mutant varied between genes, with *PKS5* showing some reduction but other genes not showing any significant reduction (Fig. 5*A*). These results indicate that SnRK2s are important for osmotic-stress-induced gene expression, with SnRK2/3/6 regulating all four genes tested, whereas SnRK1/4/5/7/8/9/10 have more specific effects on some stress-regulated genes.

To analyze genome-wide gene expression changes in the mutants, we performed microarray analysis using total RNA extracted from liquid cultured plants of wild type, *snrk2.2/3/6* triple mutant, the septuple mutant, and the decuple mutant after 3-h treatment with 0.8 M mannitol. For the wild type and the decuple mutant, RNA was also extracted from seedlings that were not treated with mannitol. First, we examined the expression of SnRK2s. The transcript level of *SnRK2.7*, which was mutated at the C-terminal end (Fig. S1 *B* and *C*), in the decuple mutant was one-third of that in the wild type (Table S1). Reduction of transcripts for other SnRK2s confirmed the mutations in the decuple mutant (Table S1). Second, we performed genome-wide comparison of gene expression in wild type and the decuple mutant after mannitol treatment.

To gain a better understanding of impact of mutations in SnRK2s in the osmotic stress response, we focused on the 464 genes induced by more than fourfold in the wild type after mannitol treatment and compared their expression between the wild type and mutants. We calculated expression in the mutants as a percentage of expression in the wild type for each gene and made histograms to show the distribution. The expression of many induced genes after the treatment was less in *snrk2.2/3/6* than in the wild type, whereas nearly all of these genes had unchanged expression (close to 100%) in the septuple mutant compared with wild type (Fig. 5*B*). In contrast, the decuple mutant was even more affected in gene expression than *snrk2.2/3/6*, with a vast majority of induced genes being expressed at lower level in the decuple mutant than in wild type or *snrk2.2/3/6.* (Fig. 5*B*). The median of the percentage expression relative to the wild type was 70–80% for *snrk2/3/6*, 100–110% for the septuple mutant, and 40–50% for the decuple mutant. Without mannitol treatment, the expression of these 464 induced genes was similar in the decuple mutant and the wild type (relative expression was close to 100%; Fig. 5*C*), indicating that the mutations did not affect the basal level of expression of the selected genes but did affect their response to osmotic stress. As a control, we quantified the expression of 464 randomly selected genes for which expression was not changed by osmotic stress in the wild type; for these noninduced genes, the expression was similar in all mutants and the wild type (relative expression was close to 100%; Fig. S3). These results indicate that the decuple *snrk2* mutant has a strong global effect on gene induction in

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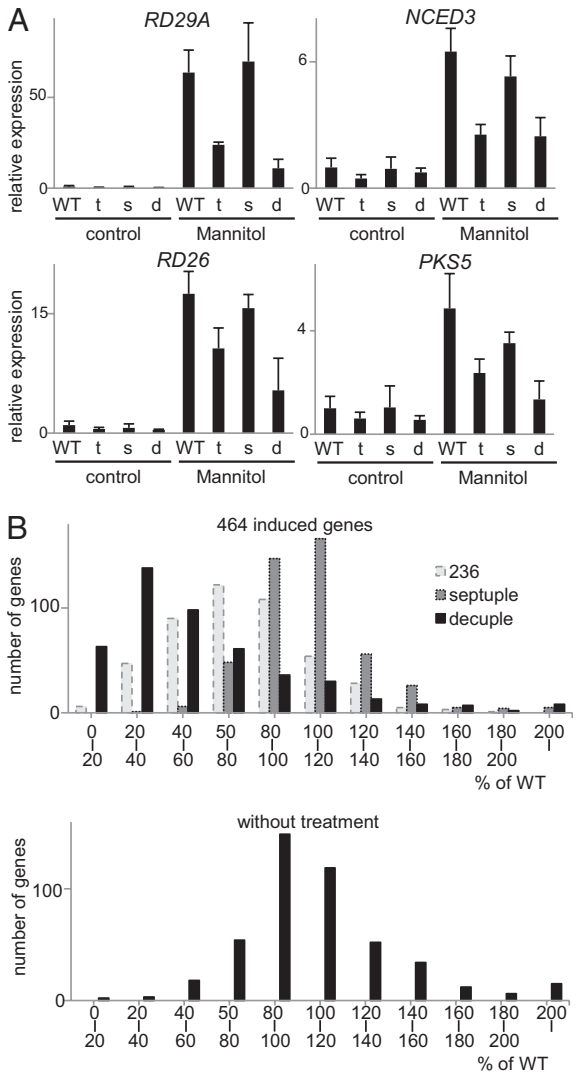

Fig. 5. Expression of genes induced by osmotic stress. (*A*) Expression of osmotic-stress–upregulated genes *RD29A*, *NCED3*, *RD26*, and *PKS5* assayed by quantitative RT-PCR in Col-0 (WT) and the *snrk2.2/3/6* triple (t), *snrk2.1/4/5/7/8/9/10* septuple (s), and *snrk2.1/2/3/4/5/6/7/8/9/10* decuple (d) mutant seedlings grown in liquid culture with or without a 3-h exposure to 0.8 M mannitol. Values are means ± SEM (n = 3 for *RD29A*, *RD26*, and *PKS5*, n = 5 for *NCED3*). (*B*) Histogram of gene expression in mutants expressed as a percentage of expression in the wild type based on microarray analysis. The bars indicate the number of genes showing the indicated relative expression, and the x axis is arranged in 11 consecutive groups (0% to <20%, ≥20% to <40%, ≥40% to <60%, —, ≥180% to <200%, and ≥200% expression relative to the wild type). (*Upper*) Expression of 464 mannitol-induced genes after mannitol treatment in the *snrk2.2/3/6*, the septuple, and the decuple mutants. (*Lower*) Expression of the same genes without mannitol treatment in the decuple mutant.

response to osmotic stress, an effect that cannot be detected even in the septuple mutant.

Decuple Mutations Affect Proline Accumulation and IP_3 Synthesis. We also analyzed other osmotic stress responses in the *snrk2* mutants. We measured proline accumulation because proline is one of the compatible osmolytes accumulated by cells under osmotic stress (42). Proline accumulation was reduced in the *snrk2.2/3/6* triple mutant and in the decuple mutant, but was greater than wild type in the septuple mutant after incubation for 12 h in liquid culture containing 0.8 M mannitol (Fig. 6*A*). In

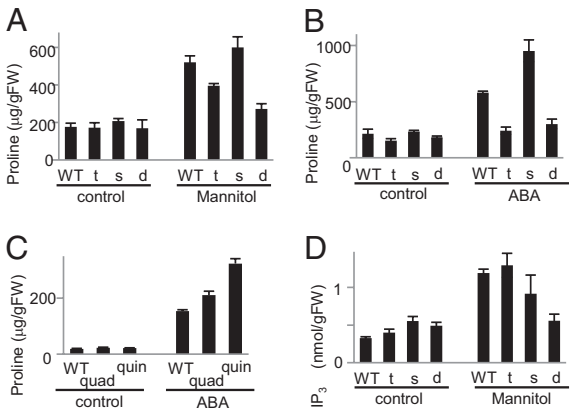

Fig. 6. Osmotic stress or ABA-induced accumulation of proline and IP_3. (*A*) Proline content measured in liquid cultured seedlings of the wild type (WT) and the snrk2.2/3/6 triple (t), the septuple (s), and the decuple (d) mutants before (control) and after a 12-h exposure to 0.8 M mannitol. Values are means ± SEM (n = 3). (*B*) Proline contents of the wild type (WT) and the *snrk2.2/3/6* triple (t), septuple (s), and decuple (d) mutants with or without a 96-h exposure to ABA on MS media plate with 3% sucrose. Values are means ± SEM (n = 3). (*C*) Proline contents of the wild type (WT) and the *snrk2.1/4/5/10* quadruple (quad) and *snrk2.1/4/5/9/10* quintuple (quin) mutants with or without a 96-h exposure to 10 μM ABA on MS media plate without sucrose. Values are means ± SEM (n = 3 for Col-0 and quadruple, n = 6 for quintuple). (*D*) IP_3 content measured in liquid culture of the wild type and the snrk2.2/3/6 triple (t), the septuple (s), and the decuple (d) mutants before (control) and after a 20-min exposure to 0.8 M mannitol. Values are means ± SEM (n = 3).

the microarray data, the expression of *P5CS1*, which is a rate-limiting factor of proline biosynthesis (43), in the triple, septuple, and decuple mutants is 65%, 112%, and 54%, respectively, of that in the wild type. This trend of proline accumulation was also observed when plants were treated with ABA (Fig. 6*B*). In addition to the enhancement in the septuple mutant, ABA-induced proline accumulation was slightly increased in the *snrk2.1/4/5/10* quadruple mutant and greatly increased in the *snrk2.1/4/5/9/10* quintuple mutant relative to the wild type (Fig. 6*C*). These results suggest that mannitol-induced proline accumulation reflects ABA-induced proline accumulation. Thus, SnRK2.2/3/6 is important for osmotic-stress-induced proline accumulation, whereas SnRK2.1/4/5/7/8/9/10 and especially SnRK2.1/4/5/9/10 may act as negative regulators of ABA-induced proline accumulation. The difference between the *snrk2.1/4/5/10* quadruple and the *snrk2.1/4/5/9/10* quintuple mutants suggests that SnRK2.9 has a negative role in ABA-induction of proline accumulation.

We also analyzed an ABA-independent response. Inositol 1,4,5-trisphosphate (IP_3) accumulation is induced by osmotic stress but not by ABA (44), although ABA induces IP_3 accumulation in a longer (≥30 min) time course (45, 46). After a 20-min exposure to 0.8 M mannitol, IP_3 accumulated in liquid culture of the wild type and *snrk2.2/3/6* but did not accumulate in the decuple mutant and was reduced in the septuple mutant (Fig. 6*D*). IP_3 accumulation was not observed under 50 μM ABA treatment for 20 min (Fig. S4). These results indicate that SnRK2s are also required for this ABA-independent response to osmotic stress.

Discussion

Although osmotic stress is an important problem in crop production, the osmotic stress signaling pathway has been largely unknown. SnRK2s have been proposed as key components of the pathway because they are activated by osmotic stress. Results of our experiments using the decuple mutant, which were impaired in all members of the SnRK2 family, clearly show the

importance of SnRK2s in the osmotic stress pathway in vivo. In the decuple mutant, all full-length mRNA of SnRK2s are disrupted (Fig. 1), resulting in elimination of the kinase activity of SnRK2s induced by osmotic stress (Fig. 2). The decuple mutants show a clear growth defect under osmotic stress, but grow as well as the wild type under nonstressed conditions (Fig. 3), indicating the essential and specific role of SnRK2s in the response of plants to osmotic stress. Disruption of normal osmotic stress response was also observed for gene expression (Fig. 5) and accumulation of ABA (Fig. 4*A*), proline (Fig. 6*A*), and IP_3 (Fig. 6*D*), all of which are important responses for stress adaptation. The decuple mutant grows poorly in soil but grows as well as the wild type on culture plates, suggesting that our soil conditions were not optimal for the decuple mutant. The roles of SnRK2.8 and SnRK2.6 in metabolism (47, 48) may also have been a factor. The observed sterility of the decuple mutant may have been caused by the high humidity, which was required for the survival of the decuple mutant plants but may have interfered with development.

SnRK2s show high redundancy. Even when nine of the genes are knocked out, the phenotype is not severe as long as SnRK2.2 is intact (Figs. 1*B* and 3). SnRK2.2 as well as SnRK2.3 and -2.6 help mediate the ABA-dependent pathway (28), and osmotic stress signaling has been considered to be divided between ABA-dependent and -independent pathways. Thus, having at least one of the ABA-activated SnRK2s is sufficient for growth and development in soil and for coping with osmotic stress in culture media. On the other hand, *snrk2.2/3/6*, which is insensitive to ABA, grew normally when subjected to osmotic stress on culture plates (Fig. 3), indicating that these ABA-activated SnRK2s are not essential for short-term osmotic stress tolerance. IP_3 accumulation, which is independent of ABA (Fig. S4*B* and ref. 44), differed between the decuple and septuple mutants (Fig. 6*D*), indicating that SnRK2.2/3/6 functions not only in the ABA-dependent pathway, but also in the ABA-independent pathway. This is consistent with the observation that SnRK2.2/3/6 is activated not only by ABA, but also by osmotic stress (Fig. 2, ref. 38). This would suggest that ABA-dependent and -independent pathways converge at the SnRK2s or their substrates.

Even in the decuple mutant, osmotic-stress-induced responses (in terms of gene expression and accumulation of ABA and proline) are greatly reduced but are not abolished, indicating that some osmotic stress signaling remains. One possibility is that the *snrk2.7* mutation may not be a null and the truncated SnRK2.7 (Fig. S1 *B* and *C*) may retain sufficient activity to trigger the observed residual responses, even though this activity is below the detection level in the in-gel kinase assay. Another possibility is that there are SnRK2-independent osmotic-stress-response pathways. Some CDPKs and SnRK3s are involved in osmotic- and salt-stress pathways (3, 49, 50). It is possible that they share some substrates with SnRK2s (51). This would result in some residual osmotic stress response in the decuple mutant, although this residual response is insufficient to rescue the impaired growth of the decuple mutant.

The results obtained with the *snrk2.1/4/5/7/8/9/10* septuple mutant are less straightforward to explain. Plants lacking SnRK2.1/4/5/9/10, which is not activated by ABA, have increased sensitivity to ABA in terms of ABA-induced proline accumulation (Fig. 6*C*). This is consistent with the finding that overexpression of a rice SnRK2.1/4/5/9/10 homolog (SAPK6) made tobacco ABA insensitive (52). The results suggest that these kinases are negative regulators of the ABA pathway. Microarray data (Fig. 5*B*) and mannitol-induced proline accumulation data (Fig. 6*A*) seem to reflect ABA responses more than ABA-independent responses. Although *snrk2.2/3/6* is more affected than the septuple mutant in terms of osmotic stress-induced gene expression, the septuple mutant is more affected than *snrk2.2/3/6* in terms of the sensitivity of growth to osmotic stress on PEG plates (Fig. 3) where the impaired stomatal control of *snrk2.2/3/6* is less of a factor than in soil-grown plants. The reduced growth of the septuple mutant could be caused by the impairment of certain key ABA-independent osmotic responses. Although the decuple mutant and the *snrk2.2/3/6* triple mutant showed similar defects in osmotic stress induction of *NCED3* expression (Fig. 5*A*), ABA deficiency was observed in the decuple mutant, but not the triple mutant (Fig. 4*A*). The results suggest that the osmotic-stress-induced ABA accumulation was not limited by the *NCED3* gene expression level but rather by other factor(s) controlled by the SnRK2.1/4/5/7/8/9/10s. The SnRK2.1/4/5/7/8/9/10s could be important for the posttranslational activation of NCED3 and other ABA biosynthesis enzymes.

Because osmotic-stress-induced kinase activity of SnRK2.9 was not detected in a protoplast assay (31), it was not clear whether SnRK2.9 plays a role in osmotic stress responses in vivo. In our results, the *snrk2.1/4/5/9/10* quintuple mutant accumulated more proline in response to ABA than the *snrk2.1/4/5/10* quadruple mutant (Fig. 6*C*). When these results are considered along with the results that ABA-induced proline accumulation of the *snrk2.9* single mutant did not significantly differ from that of the wild type ($110 \pm 8\%$, $n = 5$), they suggest that SnRK2.9 is also involved in negatively regulating the ABA pathway, but this role may be redundant with that of other SnRK2s.

Although SnRK2s have been known as osmotic stress-activated kinases, the physiological roles of the kinases in vivo have been unclear. The defects in osmotic stress responses of the *snrk2.1/2/3/4/5/6/7/8/9/10* decuple mutant revealed critical functions of these kinases in vivo.

Materials and Methods

T-DNA Insertion Lines. The *snrk2.2*, *-2.3*, and *-2.6* mutants were described in ref. 28. The seeds of *snrk2.1*, *-2.4*, *-2.5*, *-2.7*, *-2.8*, *-2.9*, and *-2.10* T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center (53). Homozygous plants were identified by PCR screening using primers designed by the I-Sect website (http://signal.salk.edu/cgi-bin/tdnaexpress; primer sequences are given in Table S2).

In-Gel Kinase Assays. In-gel kinase assays were described in ref. 27. Histone was purchased from Sigma (St. Louis, MO).

Physiological Assays. Plants in soil and seedlings in agar plates were routinely grown under 16 h of light (~75 μmol m^{-2} s^{-1}) and 8 h of dark at 23 °C.

For quantification of ABA, H_2O_2, proline, and IP_3, tissues were ground in liquid nitrogen. ABA was extracted with 80% methanol, 500 mg/L citric acid, and 10 mg/L butylated hydroxytoluene. After centrifugation to remove debris, the supernatant was dried and reconstituted with Tris-buffered saline (25 mM Tris/100 mM NaCl/1 mM $MgCl_2$, pH 7.5). ABA was measured with a Phytodetek ABA measurement kit (Agdia, Inc., Elkhart, IN) following the manufacturer's instructions. H_2O_2 was extracted with 20 mM PBS and measured by using an Amplex Red H_2O_2 kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions and ref. 40. Proline was assayed by using the ninhydrin-based colorimetric assay (54). IP_3 was assayed with an inositol-1,4,5-trisphosphate [H-3] RRA Kit (Perkin-Elmer, Waltham, MA) following the manufacturer's instructions.

RT-PCR and qPCR Analysis of Gene Expression. Total RNA was purified from seedlings by using TRIzol (Invitrogen) following the manufacturer's instructions. Reverse transcription reactions were performed using 2 μg of total RNA and SuperScriptII reverse transcriptase (Invitrogen). PCR was then performed for 35 cycles. Real-time qPCR analysis was performed with the iQ5 real-time PCR detection system and SYBR Green (Bio-Rad, Hercules, CA). PCR (15 μL) was performed with 1 μL of RT reaction. Relative amount of target mRNA was determined based on $\Delta\Delta Ct$ method using *Actin2* as an internal control. Three technical replicates were performed for each experiment. Primers used for both RT-PCR and qPCR are given in Table S2 or in ref. 27.

Microarray Analysis. Biologically duplicated total RNA was purified from seedlings by using the RNeasy plant kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Hybridization to *Arabidopsis* genome ATH1

array (Affymetrix, Santa Clara, CA) was performed by the Core Instrumental Facility of the Institute for the Integrative Genome Biology, the University of California, Riverside. Data were analyzed with affylmGUI (55).

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1. Léon-Kloosterziel K-M, et al. (1996) Isolation and characterization of abscisic acid-deficient Arabidopsis mutants at two new loci. *Plant J* 10:655–661.
2. Chow B, McCourt P (2004) Hormone signalling from a developmental context. *J Exp Bot* 55:247–251.
3. Zhu J-K (2002) Salt and drought stress signal transduction in plants. *Annu Rev Plant Biol* 53:247–273.
4. Finkelstein R-R, Reeves W, Ariizumi T, Steber C (2008) Molecular aspects of seed dormancy. *Annu Rev Plant Biol* 59:387–415.
5. Acharya BR, Assmann SM (2009) Hormone interactions in stomatal function. *Plant Mol Biol* 69:451–462.
6. Kim T-H, Böhmer M, Hu H, Nishimura N, Schroeder J-I (2010) Guard cell signal transduction network: advances in understanding abscisic acid, CO2, and Ca2+ signaling. *Annu Rev Plant Biol* 61:561–591.
7. Park S-Y, et al. (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* 324:1068–1071.
8. Ma Y, et al. (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* 324:1064–1068.
9. Umezawa T, et al. (2009) Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proc Natl Acad Sci USA* 106:17588–17593.
10. Vlad F, et al. (2009) Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in Arabidopsis. *Plant Cell* 21:3170–3184.
11. Fujii H, et al. (2009) In vitro reconstitution of an abscisic acid signalling pathway. *Nature* 462:660–664.
12. Kobayashi Y, et al. (2005) Abscisic acid-activated SNRK2 protein kinases function in the gene-regulation pathway of ABA signal transduction by phosphorylating ABA response element-binding factors. *Plant J* 44:939–949.
13. Furihata T, et al. (2006) Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. *Proc Natl Acad Sci USA* 103:1988–1993.
14. Piskurewicz U, et al. (2008) The gibberellic acid signaling repressor RGL2 inhibits Arabidopsis seed germination by stimulating abscisic acid synthesis and ABI5 activity. *Plant Cell* 20:2729–2745.
15. Geiger D, et al. (2009) Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. *Proc Natl Acad Sci USA* 106:21425–21430.
16. Lee S-C, Lan W, Buchanan B-B, Luan S (2009) A protein kinase-phosphatase pair interacts with an ion channel to regulate ABA signaling in plant guard cells. *Proc Natl Acad Sci USA* 106:21419–21424.
17. Sirichandra C, et al. (2009) Phosphorylation of the Arabidopsis AtrbohF NADPH oxidase by OST1 protein kinase. *FEBS Lett* 583:2982–2986.
18. Sato A, et al. (2009) Threonine at position 306 of the KAT1 potassium channel is essential for channel activity and is a target site for ABA-activated SnRK2/OST1/SnRK2.6 protein kinase. *Biochem J* 424:439–448.
19. Verslues P-E, Zhu J-K (2007) New developments in abscisic acid perception and metabolism. *Curr Opin Plant Biol* 10:447–452.
20. Xiong L-M, Lee H-J, Ishitani M, Zhu J-K (2002) Regulation of osmotic stress-responsive gene expression by the LOS6/ABA1 locus in Arabidopsis. *J Biol Chem* 277:8588–8596.
21. Barrero J-M, et al. (2006) Both abscisic acid (ABA)-dependent and ABA-independent pathways govern the induction of *NCED3, AAO3* and *ABA1* in response to salt stress. *Plant Cell Environ* 29:2000–2008.
22. Hrabak E-M, et al. (2003) The Arabidopsis CDPK-SnRK superfamily of protein kinases. *Plant Physiol* 132:666–680.
23. Anderberg R-J, Walker-Simmons M-K (1992) Isolation of a wheat cDNA clone for an abscisic acid-inducible transcript with homology to protein kinases. *Proc Natl Acad Sci USA* 89:10183–10187.
24. Li J, Wang X-Q, Watson M-B, Assmann S-M (2000) Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. *Science* 287:300–303.
25. Mustilli A-C, Merlot S, Vavasseur A, Fenzi F, Giraudat J (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* 14:3089–3099.
26. Yoshida R, et al. (2002) ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in Arabidopsis. *Plant Cell Physiol* 43:1473–1483.
27. Fujii H, Verslues P-E, Zhu J-K (2007) Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in Arabidopsis. *Plant Cell* 19:485–494.
28. Fujii H, Zhu J-K (2009) Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. *Proc Natl Acad Sci USA* 106:8380–8385.
29. Fujita Y, et al. (2009) Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in Arabidopsis. *Plant Cell Physiol* 50:2123–2132.
30. Nakashima K, et al. (2009) Three Arabidopsis SnRK2 protein kinases, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. *Plant Cell Physiol* 50:1345–1363.
31. Boudsocq M, Barbier-Brygoo H, Laurière C (2004) Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in Arabidopsis thaliana. *J Biol Chem* 279:41758–41766.
32. Mikołajczyk M, Awotunde O-S, Muszyńska G, Klessig D-F, Dobrowolska G (2000) Osmotic stress induces rapid activation of a salicylic acid-induced protein kinase and a homolog of protein kinase ASK1 in tobacco cells. *Plant Cell* 12:165–178.
33. Monks D-E, Aghoram K, Courtney P-D, DeWald D-B, Dewey R-E (2001) Hyperosmotic stress induces the rapid phosphorylation of a soybean phosphatidylinositol transfer protein homolog through activation of the protein kinases SPK1 and SPK2. *Plant Cell* 13:1205–1219.
34. Kobayashi Y, Yamamoto S, Minami H, Kagaya Y, Hattori T (2004) Differential activation of the rice sucrose nonfermenting1-related protein kinase2 family by hyperosmotic stress and abscisic acid. *Plant Cell* 16:1163–1177.
35. Umezawa T, Yoshida R, Maruyama K, Yamaguchi-Shinozaki K, Shinozaki K (2004) SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling stress-responsive gene expression in Arabidopsis thaliana. *Proc Natl Acad Sci USA* 101:17306–17311.
36. Mao X, Zhang H, Tian S, Chang X, Jing R (2010) TaSnRK2.4, an SNF1-type serine/threonine protein kinase of wheat (Triticum aestivum L.), confers enhanced multistress tolerance in Arabidopsis. *J Exp Bot* 61:683–696.
37. Mizoguchi M, et al. (2010) Two closely related subclass II SnRK2 protein kinases cooperatively regulate drought-inducible gene expression. *Plant Cell Physiol* 51:842–847.
38. Boudsocq M, Droillard M-J, Barbier-Brygoo H, Laurière C (2007) Different phosphorylation mechanisms are involved in the activation of sucrose non-fermenting 1 related protein kinases 2 by osmotic stresses and abscisic acid. *Plant Mol Biol* 63:491–503.
39. Verslues P-E, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu J-K (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant J* 45:523–539.
40. Verslues PE, et al. (2007) Interaction of SOS2 with nucleoside diphosphate kinase 2 and catalases reveals a point of connection between salt stress and H2O2 signaling in Arabidopsis thaliana. *Mol Cell Biol* 27:7771–7780.
41. Takahashi S, et al. (2004) Monitoring the expression profiles of genes induced by hyperosmotic, high salinity, and oxidative stress and abscisic acid treatment in Arabidopsis cell culture using a full-length cDNA microarray. *Plant Mol Biol* 56:29–55.
42. Yoshiba Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K (1997) Regulation of levels of proline as an osmolyte in plants under water stress. *Plant Cell Physiol* 38:1095–1102.
43. Yoshiba Y, et al. (1995) Correlation between the induction of a gene for delta 1-pyrroline-5-carboxylate synthetase and the accumulation of proline in Arabidopsis thaliana under osmotic stress. *Plant J* 7:751–760.
44. Takahashi S, Katagiri T, Hirayama T, Yamaguchi-Shinozaki K, Shinozaki K (2001) Hyperosmotic stress induces a rapid and transient increase in inositol 1,4,5-trisphosphate independent of abscisic acid in Arabidopsis cell culture. *Plant Cell Physiol* 42:214–222.
45. Sanchez J-P, Chua N-H (2001) Arabidopsis PLC1 is required for secondary responses to abscisic acid signals. *Plant Cell* 13:1143–1154.
46. Burnette R-N, Gunesekera B-M, Gillaspy G-E (2003) An Arabidopsis inositol 5-phosphatase gain-of-function alters abscisic acid signaling. *Plant Physiol* 132:1011–1019.
47. Shin R, Alvarez S, Burch A-Y, Jez J-M, Schachtman D-P (2007) Phosphoproteomic identification of targets of the Arabidopsis sucrose nonfermenting-like kinase SnRK2.8 reveals a connection to metabolic processes. *Proc Natl Acad Sci USA* 104:6460–6465.
48. Zheng Z, et al. (2010) The protein kinase SnRK2.6 mediates the regulation of sucrose metabolism and plant growth in Arabidopsis. *Plant Physiol* 153:99–113.
49. Sheen J (1996) Ca2+-dependent protein kinases and stress signal transduction in plants. *Science* 274:1900–1902.
50. Liu J, Ishitani M, Halfter U, Kim C-S, Zhu J-K (2000) The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance. *Proc Natl Acad Sci USA* 97:3730–3734.
51. Vlad F, Turk B-E, Peynot P, Leung J, Merlot S (2008) A versatile strategy to define the phosphorylation preferences of plant protein kinases and screen for putative substrates. *Plant J* 55:104–117.
52. Chae M-J, et al. (2007) A rice dehydration-inducible SNF1-related protein kinase 2 phosphorylates an abscisic acid responsive element-binding factor and associates with ABA signaling. *Plant Mol Biol* 63:151–169.
53. Alonso J-M, et al. (2003) Genome-wide insertional mutagenesis of *Arabidopsis* thaliana. *Science* 301:653–657.
54. Bates L-S, Waldren R-P, Teare I-D (1973) Rapid determination of free proline in water-stress studies. *Plant Soil* 39:205–207.
55. Smyth G-K (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology* 3(1): Article 3. Available at http://www.bepress.com/sagmb/vol3/iss1/art3/.